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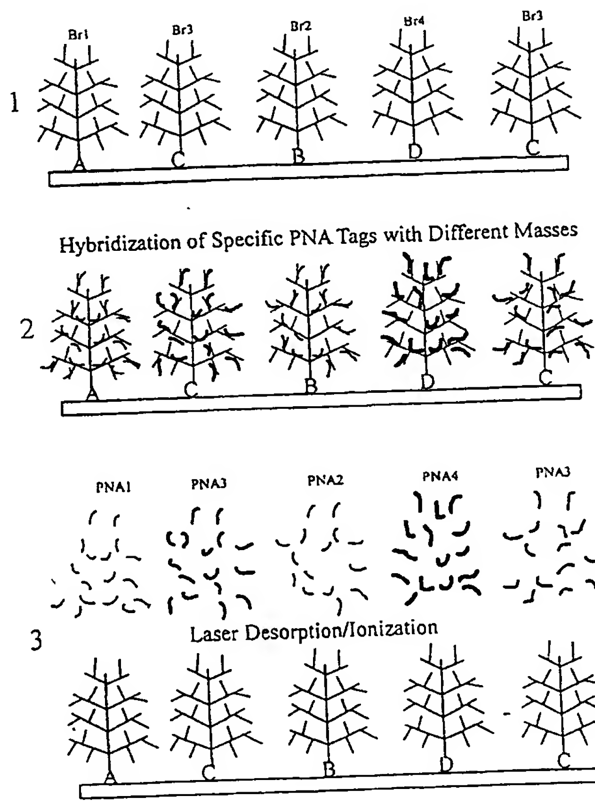
(54) Title: MULTIPLE TAG ANALYSIS

(57) Abstract

Disclosed is a method of detecting multiple analytes in a sample in a single assay. The method is based on encoding target molecules with signals followed by decoding of the encoded signal. This encoding/decoding uncouples the detection of a target molecule from the chemical and physical properties of the target molecule. In basic form, the disclosed method involves association of one or more reporter molecules with one or more target samples, association of one or more decoding tags with the reporter molecules, and detection of the decoding tags. The reporter molecules associate with target molecules in the target sample(s). Generally, the reporter molecules correspond to one or more target molecules, and the decoding tags correspond to one or more reporter molecules. Thus, detection of particular decoding tags indicates the presence of the corresponding reporter molecules. In turn, the presence of particular reporter molecules indicates the presence of the corresponding target molecules. The sensitivity of the disclosed method can also be enhanced by including a signal amplification step prior to detection. Medical applications of this method include the analysis of the phenotypic status or replicative status of cells (growth or quiescence) and the assessment of normal and neoplastic cells in histologic or cytologic specimens in normal and disease states. For example, a pathologist may use the method to link a phenotypic state with the protein profile of lesion believed to contain malignant or pre–malignant cells.



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MULTIPLE TAG ANALYSIS

BACKGROUND OF THE INVENTION

The present invention is generally in the field of detection of molecules, and specifically in the field of detection of multiple different molecules in a single assay.

The analysis of proteins in histological sections and other cytological preparations is routinely performed using the techniques of histochemistry, immunohistochemistry, or immunofluorescence. By performing immunofluorescence with antibodies labeled with different colors, it has been possible to detect simultaneously 2, 3, or even 4 different antigens present in cellular material. In the future, time-resolved fluorescence may permit the extension of immunofluorescence methods to the detection of 6 to 12 different antibodies simultaneously. Likewise, RNA detection by fluorescence in situ hybridization permits the detection of 2 to 4 different RNAs in cellular material, and it may also be extended to permit the detection of 6 to 12 different RNAs by time-resolved fluorescence.

There is a need for a sensitive method that will permit the cytological detection of larger numbers of proteins or RNAs simultaneously. Theoretically, the simultaneous measurement of the concentration of 20 to 50 different protein (or RNA) species should be highly informative as to the specific status of dynamic cellular processes in normal development, in stages of disease, in response to drug treatment or gene therapy, or as a result of environmental exposure or other deliberate or inadvertent interventions.

The study of cells by measuring the identity and concentration of a relatively large number of proteins simultaneously (referred to as proteomics) is currently a very time-consuming task. Two-dimensional (2D) gel electrophoresis is the most powerful tool for studying the expression of multiple proteins, but this technique is not readily adaptable to in-situ cell analysis. Typically, many thousands of cells are required to perform a single 2D gel analysis. In order to identify different protein expression profiles in heterogeneous tissue samples, one would need the capability to analyze the proteins expressed in a small number of cells. This capability is most relevant in the analysis of histological or cytological specimens that may harbor dysplastic or pre-malignant cells. Such

cells, which may precede the development of cancer, need to be identified when present as small foci of 10 to 50 cells, before they have a chance to give rise to tumors. Unfortunately, the amount of protein obtained from 10 to 50 cells is insufficient for 2D gel analysis, and is problematic even with the use of radioisotopes to label the protein.

Mass spectroscopy is another powerful technique for protein analysis. However, the direct analysis of proteins present in samples containing small numbers of cells is not possible with prior mass spectroscopy technology, due to insufficient sensitivity. A minimum of 10,000 cells is required for mass spectroscopic analysis of tissue samples using prior technology.

Current methods for the analysis of microarray hybridization experiments rely on the use of a two-color signal readout system. For example, Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467-70, describe an experiment where cDNA prepared from one tissue is labeled with the dye cy3, while cDNA from another tissue is labeled with the dye cy5. After the labeling reactions are performed, the two labeled DNAs are mixed, and hybridized by contacting with the surface of a glass slide containing a cDNA microarray on its surface. At the end of the hybridization reaction, the microarray surface is washed to remove unhybridized material, and the glass slide is scanned in a confocal scanning instrument designed to record separately the cy3 and the cy5 fluorescence intensity, which is saved as two different computer files. Computer software is then used to calculate the fluorescence ratio of cy3 to cy5 at each of the specific dot-addresses on the DNA microarray. This experimental design works very well for performing comparisons of mRNA expression ratios between two samples.

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nature Biotechnology* 17:994-999, have described an approach for the accurate quantification and concurrent sequence identification of the individual proteins within complex mixtures of biological origin. The method is based on a class of new chemical reagents termed isotope-coded affinity tags (ICATs), and tandem mass spectrometry. These authors extracted proteins from two different

experimental states of an organism, and labeled each of the two preparations of total protein with two different thiol-reactive ICAT tags of different mass. The two labeled protein preparations were mixed, separated by liquid chromatography, and detected on line by mass spectrometry. For each individual protein peak, mass spectrometry permitted protein identification, as well as measurement of the ratio of the amounts of the two proteins.

In pioneering work, Ekins and Chu (Ekins, R.P. and Chu, F.W. (1991) Multianalyte microspot immunoasay – Microanalytical "Compact Disk" of the future. Clinical Chemistry 37:1955-1967; Ekins, R.P. and Chu, F.W. (1994) Developing multianalyte assays Trends in Biotechnology 12:89-94) described assays in which different specific antibodies are laid down on a surface as an array of microspots, subsequently a complex sample containing a mixture of antigens is bound to the array, and a second antibody is used for detection. In this multiplex assay, the signal present at each address serves as an indicator of the concentration of each of a multiplicity of antigens present in the sample. The authors suggest that these microarrays could contain as many as 10,000 antibody microspots per square centimeter.

However, a serious limitation of all existing microarray methods, including those disclosed above, is that they do not permit the analysis of more than six to eight samples in a single microarray experiment. If fluorescent dyes are used for detection, there are problems of spectral overlap of multiple dyes if more than seven dyes are used. If non-amplifiable mass tags are used, there is insufficient sensitivity of the mass spectrometric detection step, due to the fact that the total number of protein molecules bound on each antibody spot of the microarray is relatively small (typically 20,000 to 1,000,000 protein molecules per spot, for a simple two-sample experiment, or even less for a multi-sample experiment).

It is therefore an object of the present invention to provide a method that permits the indirect detection of a large number of different analytes in a single sample or group of samples.

It is therefore another object of the present invention to provide a method that permits the indirect detection of a large number of different proteins in a single sample or group of samples.

It is another object of the present invention to provide a method that permits the indirect detection of a large number of different mRNAs in a single sample or group of samples.

It is another object of the present invention to provide a method of detecting indirectly modification states of a multiple analytes in a single sample or group of samples.

BRIEF SUMMARY OF THE INVENTION

Disclosed is a method of detecting multiple analytes in a sample in a single assay. The method is based on encoding target molecules with signals followed by decoding of the encoded signal. This encoding/decoding uncouples the detection of a target molecule from the chemical and physical properties of the target molecule. In basic form, the disclosed method involves association of one or more reporter molecules with one or more target samples, association of one or more decoding tags with the reporter molecules, and detection of the decoding tags. The reporter molecules associate with target molecules in the target sample(s). Generally, the reporter molecules correspond to one or more target molecules, and the decoding tags correspond to one or more reporter molecules. Thus, detection of particular decoding tags indicates the presence of the corresponding reporter molecules. In turn, the presence of particular reporter molecules indicates the presence of the corresponding target molecules.

This indirect detection uncouples the detection of target molecules from the chemical and physical properties of the target molecules by interposing decoding tags that essentially can have any arbitrary chemical and physical properties. In particular, decoding tags can have specific properties useful for detection, and decoding tags within an assay can have highly ordered or structured relationships with each other. It is the (freely chosen) properties of the decoding tags, rather than the (take them as they are) properties of the target molecules that matters at the point of detection.

The decoding tags have the additional advantage of being uncoupled from the target molecule-specific aspects of the reporter molecules. Unlike detection methods where a labeled molecule is bound to an analyte followed by detection of the label, the disclosed method is not limited by the chemical and physical properties of the labeled molecule. This allows more convenient

detection, more sensitive detection, and more highly multiplexed detection schemes.

The sensitivity of the disclosed method can also be enhanced by including a signal amplification step prior to detection. In basic form, amplification is accomplished by amplifying reporter signals on the reporter molecules. This results in multiple reporter tags associated with each reporter molecule. The decoding tags are then associated with the reporter tags and detected. Generally, the decoding tags correspond to one or more reporter tags (and thus correspond to the reporter molecules with which the reporter tags are associated), and the reporter tags correspond to the reporter molecules with which they are associated. Thus, detection of particular decoding tags indicates the presence of the corresponding reporter tags. In turn, the presence of particular reporter tags indicates the presence of the corresponding reporter molecules. In turn, the presence of particular reporter molecules indicates the presence of the corresponding target molecules. The reporter molecule also can be designed to include multiple reporter tags (essentially accomplishing a pre-assay amplification of the signal).

In one form of the disclosed method, one or more target samples and one or more reporter molecules are brought into contact, allowing the reporter molecules to become associated with target molecules in the target samples. The reporter molecules are then amplified to produce multiple reporter tags for each reporter molecule. The reporter tags remain associated with the reporter molecules. One or more decoding tags are then associated with the reporter tags, and the decoding tags are detected. The decoding tags correspond to reporter molecules and the reporter molecules correspond to target molecules. This relationship means that detecting the decoding tags indicates the presence of reporter molecules corresponding to the detected decoding tags, and that the presence of reporter molecules indicates the presence of target molecules corresponding to the reporter molecules.

In another form of the disclosed method, four or more target samples and one or more reporter molecules are brought into contact, allowing the reporter molecules become associated with target molecules in the target samples. Each target sample is brought into contact with a different set of reporter molecules,

and the reporter molecules in each set of reporter molecules are different from the reporter molecules in the other sets of reporter molecules. The four or more of the target samples are then mixed together, one or more decoding tags are associated with the reporter molecules, and the decoding tags are detected. A different decoding tag corresponds to each different reporter molecule such that each decoding tag corresponds to only one of the target samples. This relationship means that detecting the decoding tags indicates the presence of target molecules corresponding to the detected decoding tags. Further, detection of decoding tags corresponding to different target samples indicates the presence of the same target molecules in the corresponding target samples.

In another form of the disclosed method, one or more target samples and one or more reporter molecules are brought into contact, allowing the reporter molecules to become associated with target molecules in the target samples. One or more decoding tags are then associated with the reporter molecules. A different decoding tag corresponds to each different reporter molecule, and the decoding tags are not covalently coupled to the reporter molecules. The decoding tags are then detected by disassociating the decoding tags from the reporter molecules. The decoding tags correspond to reporter molecules and the reporter molecules correspond to target molecules. This relationship means that detecting the decoding tags indicates the presence of reporter molecules corresponding to the detected decoding tags, and that the presence of reporter molecules indicates the presence of target molecules corresponding to the reporter molecules.

In another form of the disclosed method, one or more target samples and one or more reporter carriers are brought into contact. The reporter carriers include one or more specific binding molecules, a carrier, and a plurality of decoding tags associated with the carrier. After the reporter carriers are associated with reporter molecules, the decoding tags are detected. The decoding tags correspond to reporter carriers, and the reporter carriers correspond to target molecules. This relationship means that detecting the decoding tags indicates the presence of reporter carriers corresponding to the detected decoding tags, and that the presence of reporter carriers indicates the presence of target molecules corresponding to the reporter carriers.

In preferred embodiments, a one-to-one-to-one correspondence between target molecules, reporter molecules, and decoding tags is used. In this way, each different type of target molecule ends up with a different and separately detectable decoding tag associated with it. The combination of specific binding molecule and reporter signal results in an effective conversion of the different target molecules (where the target molecules may be widely chemically divergent) into a standardized signal (the reporter signal). These standardized signals (which are coded for each target molecule) are then detected using a single set of standardized conditions (during which the coding is carried forward). The detection of the coded signals (that is, the decoding tags) results in effective, convenient detection of multiple target molecules in a single assay.

Decoding tags can be detected using any suitable technique. In general, the properties of the decoding tags will be chosen to match, or be compatible with, a chosen detection technique. In preferred embodiments, the disclosed method uses rapid detection techniques that allow spatial information about analytes to be gathered. An example is matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy and electrophoresis (preferably used in conjunction with microdissection). Such techniques allow automation and rapid throughput of multiple samples and assays.

This disclosed method enables a multiplex approach to the study of proteins and other analytes in microscopic tissue specimens. Compared to immunofluorescence, the disclosed method offers a greatly increased capability for multiplexing. The method is also useful for *in situ* mRNA profiling. The disclosed method permits the indirect detection of a large number of different proteins (from 20 to 50, for example, limited only by the number of specific antibodies available).

Medical applications of this method include the analysis of the phenotypic status of cells (growth or quiescence) and the assessment of normal and neoplastic cells in histologic or cytologic specimens in normal and disease states. For example, a pathologist may use the method to link a phenotypic state with the protein profile of a lesion believed to contain malignant or pre-malignant cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram illustrating and example of the disclosed method using branched DNA for amplification. Section 1 shows different branched DNA molecules (Br1, Br2, Br3, and Br4) associated with their cognate reporter signals (A, B, C, and D, respectively). The reporter signals are associated (via specific binding molecules to which they are coupled to form reporter molecules) to target molecules on a surface. Section 2 shows peptide nucleic acid decoding tags (PNA1, PNA2, PNA3, and PNA4) hybridized to their cognate reporter tags on the associated branched DNA molecules. Section 3 shows laser desorption/ionization of the decoding tags for analysis by mass spectroscopy.

Figure 2 is a diagram illustrating an example of the disclosed method using branched DNA for amplification. Section 1 shows different branched DNA molecules (Br1, Br2, Br3, and Br4) associated with their cognate reporter signals. The reporter signals are associated--via specific binding molecules (antibodies A, B, C, and D) to which they are coupled to form reporter molecules--to target molecules on a surface (antigens a, b, c, and d). Section 2 shows peptide nucleic acid decoding tags (PNA1, PNA2, PNA3, and PNA4) hybridized to their cognate reporter tags on the associated branched DNA molecules. Section 3 shows laser desorption/ionization of the decoding tags for analysis by mass spectroscopy.

Figure 3 is a diagram illustrating an example of the disclosed method using branched DNA for amplification. Section 1 shows different branched DNA molecules (Br1, Br2, Br3, and Br4) associated with their cognate reporter signals. The reporter signals are associated--via specific binding molecules (antigens a, b, c, and d) to which they are coupled to form reporter molecules--to target molecules on a surface (antibodies A, B, C, and D). Section 2 shows peptide nucleic acid decoding tags (PNA1, PNA2, PNA3, and PNA4) hybridized to their cognate reporter tags on the associated branched DNA molecules. Section 3 shows laser desorption/ionization of the decoding tags for analysis by mass spectroscopy.

Figure 4 is a diagram illustrating an example of the disclosed method using branched DNA for amplification. Section 1 shows different branched

DNA molecules (Br1, Br2, Br3, and Br4) associated with their cognate reporter signals. The reporter signals (', ", "', and "") are associated--via specific binding molecules to which they are coupled to form reporter molecules--to target molecules (antigen a). The target molecules are immobilized on a surface via interaction with a capture tags on the surface (antibody A). The different reporter signals are associated with the same target molecules in different target samples. Section 2 shows peptide nucleic acid decoding tags (PNA1, PNA2, PNA3, and PNA4) hybridized to their cognate reporter tags on the associated branched DNA molecules. Section 3 shows laser desorption/ionization of the decoding tags for analysis by mass spectroscopy.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed is a method using a specific signal decoding technique for identifying and measuring the concentration of different target molecules in an assay. The method is especially useful for assays carried out on substrates such as plates, surfaces, slides, and beads. The method is particularly suited for the detection of macromolecular analytes present in cells or tissues. For example, the disclosed method is useful for correlating phenotypic status with particular analytes in cell or tissue samples.

One form of the disclosed method is based on specific recognition of each analyte, amplification of the recognition event, and detection of the amplified signal. The specific recognition is accomplished with specific binding molecules that bind or otherwise interact specifically with an analyte of interest. The amplification of this interaction is mediated by a reporter signal coupled, tethered, or otherwise attached to the specific binding molecule. It is the reporter signal, an oligonucleotide, that is used to produce multiple reporter tags that remain associated with the reporter signal (and thus also with the specific binding molecule and analyte). This is accomplished, for example, by amplification of the reporter signal or hybridization of branched DNA or oligonucleotide dendrimers to the reporter signals. Detector tags, which are coded to include distinct, separately detectable attributes, are then hybridized to the reporter tags. Detection of the various detector tags results in indirect detection of the various analytes in the sample.

In the disclosed method, multiple analytes (which may be proteins, nucleic acid molecules, or other molecules that can be specifically recognized) are assayed using a signal amplification system capable of generating multiple copies of an oligonucleotide (called a reporter tag), where the reporter molecule is physically anchored at or associated with a specific site on the solid surface; specifically, at the site where an analyte is located. An example of such a signal amplification system is branched DNA (Urdea, *Biotechnology* 12:926-928 (1994); Horn *et al., Nucleic Acids Res* 23:4835-4841 (1997)).

In a preferred embodiment, peptide nucleic acid (PNA) molecules (Hanvey et al., Science 258:1481-1485 (1992)) of different sequence and molecular weight are used as arbitrary decoding tags that bind specifically to unique reporter tags. Laser desorption occurring on specific areas of the cytological samples is used to generate matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra of the PNA tags, which are released into the spectrometer and resolved by mass. The intensity of each PNA decoding tag reveals the relative amount of different DNA reporters. In other words, the PNA spectra generate scalar values that are indirect indicators of the relative abundance of the analytes of interest at specific locations on the glass surface.

For protein detection, for example, suitable reporter molecules include a specific antibody (specific binding molecules) covalently coupled to a DNA oligonucleotide (reporter signal). Each antibody is specific for a protein antigen of interest. The covalently coupled oligonucleotide includes a specific DNA sequence that serves to identify the antibody, and furthermore serves to generate a signal comprising amplified, surface-localized DNA. An example of a surface-localized DNA signal of amplified DNA is the branched DNA technology described in Urdea, Biotechnology 12:926 (1994), and Hong et al., Nucleic Acids Res 23:4835-4841 (1997). Another example of a surface-localized DNA signal is the tandemly repeated DNA generated by rolling circle reporter systems (Lizardi et al., *Nature Genetics* 19:225-232 (1998); U.S. Patent No. 5,854,033 to Lizardi; PCT Application No. WO 97/19193). In the case where branched DNA is used for amplification, the DNA oligonucleotide that is bound to each antibody serves to initiate the formation of a branched DNA

structure. Alternatively, the DNA oligonucleotide that is bound to each antibody can serve to initiate rolling circle replication (by serving as a primer, for example), generating a large tandem DNA molecule that remains localized at the site of antibody binding.

In another embodiment, referred to as multiple electrophoretic tag assay (META), detector tags that are sequence-coded, size-coded, and fluorescently-labeled are detected by capillary electrophoresis. Capillary electrophoresis coupled with laser-induced fluorescence is used to generate a quantitative profile of the different decoding tags. The intensity of each fluorescent peak reveals the relative amount of each different decoding tags. In other words, the peak sizes of the different decoding tags are scalar values that are indirect indicators of the relative abundance of the analytes of interest in the sample.

Materials

Reporter Molecules

Reporter molecules are molecules that combine a specific binding molecule with a reporter signal. Preferably, the specific binding molecule and reporter signal are covalent coupled or tethered to each other. As used herein, molecules are coupled when they are covalent joined, directly or indirectly. One form of indirect coupling is via a linker molecule. The reporter signal can be coupled to the specific binding molecule by any of several established coupling reactions. For example, Hendrickson *et al.*, *Nucleic Acids Res.*, **23**(3):522-529 (1995) describes a suitable method for coupling oligonucleotides to antibodies.

As used herein, a molecule is said to be tethered to another molecule when a loop of (or from) one of the molecules passes through a loop of (or from) the other molecule. The two molecules are not covalently coupled when they are tethered. Tethering can be visualized by the analogy of a closed loop of string passing through the hole in the handle of a mug. In general, tethering is designed to allow one or both of the molecules to rotate freely around the loop.

Specific Binding Molecules

A specific binding molecule is a molecule that interacts specifically with a particular molecule or moiety. The molecule or moiety that interacts specifically with a specific binding molecule is referred to herein as a target molecule. It is to be understood that the term target molecule refers to both

separate molecules and to portions of such molecules, such as an epitope of a protein, that interacts specifically with a specific binding molecule. Antibodies, either member of a receptor/ligand pair, synthetic polyamides (Dervan, P.B. and R.W. Burli, *Sequence-specific DNA recognition by polyamides.* Curr Opin Chem Biol, 1999. 3(6): p. 688-93. Wemmer, D.E. and P.B. Dervan, *Targeting the minor groove of DNA.* Curr Opin Struct Biol, 1997. 7(3): p. 355-61.), and other molecules with specific binding affinities are examples of specific binding molecules, useful as the affinity portion of a reporter binding molecule.

A specific binding molecule that interacts specifically with a particular target molecule is said to be specific for that target molecule. For example, where the specific binding molecule is an antibody that binds to a particular antigen, the specific binding molecule is said to be specific for that antigen. The antigen is the target molecule. The reporter molecule containing the specific binding molecule can also be referred to as being specific for a particular target molecule. Specific binding molecules preferably are antibodies, ligands, binding proteins, receptor proteins, haptens, aptamers, carbohydrates, synthetic polyamides, or oligonucleotides. Preferred binding proteins are DNA binding proteins. Preferred DNA binding proteins are zinc finger motifs, leucine zipper motifs, helix-turn-helix motifs. These motifs can be combined in the same specific binding molecule.

Antibodies useful as the affinity portion of reporter binding agents, can be obtained commercially or produced using well established methods. For example, Johnstone and Thorpe, *Immunochemistry In Practice* (Blackwell Scientific Publications, Oxford, England, 1987) on pages 30-85, describe general methods useful for producing both polyclonal and monoclonal antibodies. The entire book describes many general techniques and principles for the use of antibodies in assay systems.

Properties of zinc fingers, zinc finger motifs, and their interactions, are described by Nardelli, J., T. Gibson, and P. Charnay, *Zinc finger-DNA recognition: analysis of base specificity by site- directed mutagenesis.* Nucleic Acids Res, 1992. **20**(16): p. 4137-44, Jamieson, A.C., S.H. Kim, and J.A. Wells; *In vitro selection of zinc fingers with altered DNA-binding specificity.* Biochemistry, 1994. **33**(19): p. 5689-95, Chandrasegaran, S. and J. Smith,

Chimeric restriction enzymes: what is next? Biol Chem, 1999. **380**(7-8): p. 841-8, and Smith, J., J.M. Berg, and S. Chandrasegaran, *A detailed study of the substrate specificity of a chimeric restriction enzyme.* Nucleic Acids Res, 1999. **27**(2): p. 674-81.

One form of specific binding molecule is an oligonucleotide or oligonucleotide derivative. Such specific binding molecules are designed for and used to detect specific nucleic acid sequences. Thus, the target molecule for oligonucleotide specific binding molecules are nucleic acid sequences. The target molecule can be a nucleotide sequence within a larger nucleic acid molecule. An oligonucleotide specific binding molecule can be any length that supports specific and stable hybridization between the reporter binding probe and the target molecule. For this purpose, a length of 10 to 40 nucleotides is preferred, with an oligonucleotide specific binding molecule 16 to 25 nucleotides long being most preferred. It is preferred that the oligonucleotide specific binding molecule is peptide nucleic acid. Peptide nucleic acid forms a stable hybrid with DNA. This allows a peptide nucleic acid specific binding molecule to remain firmly adhered to the target sequence during subsequent amplification and detection operations.

This useful effect can also be obtained with oligonucleotide specific binding molecules by making use of the triple helix chemical bonding technology described by Gasparro *et al.*, *Nucleic Acids Res.* 1994 22(14):2845-2852 (1994). Briefly, the oligonucleotide specific binding molecule is designed to form a triple helix when hybridized to a target sequence. This is accomplished generally as known, preferably by selecting either a primarily homopurine or primarily homopyrimidine target sequence. The matching oligonucleotide sequence which constitutes the specific binding molecule will be complementary to the selected target sequence and thus be primarily homopyrimidine or primarily homopurine, respectively. The specific binding molecule (corresponding to the triple helix probe described by Gasparro *et al.*) contains a chemically linked psoralen derivative. Upon hybridization of the reporter binding probe to a target sequence, a triple helix forms. By exposing the triple helix to low wavelength ultraviolet radiation, the psoralen derivative mediates cross-linking of the probe to the target sequence.

Reporter Signals

Reporter signals are molecules or moieties that form part of a reporter molecule and that allow amplification or detection of the reporter molecule. Reporter signals can have any structure that allows either amplification of their signal or association with a decoding tag. A single reporter signal is a single signal. Amplification of this single signal results in production of multiple signals (which are referred to as reporter tags). As discussed elsewhere herein, this amplification is not limited to, but can include, actual amplification (that is, replication) of the reporter signal. Preferably, the reporter signal is designed to serve as a hybridization partner (to associate, for example, branched DNA or oligonucleotide dendrimers with the probe) or as a DNA synthesis primer.

In one embodiment, the reporter signal is an oligonucleotide and includes a sequence that serves as a hybridization partner for the tail of a branched DNA molecule or of an oligonucleotide dendrimer. The sequence of the probe sequence can be arbitrarily chosen. Where multiple oligonucleotide reporter molecules are being used in the same assay (the normal situation), it is preferred that the reporter signal sequence for each reporter molecule be substantially different to limit the possibility of non-specific target detection.

In another embodiment, the oligonucleotide portion of a reporter molecule includes a sequence, referred to as a rolling circle replication primer sequence, that serves as a rolling circle replication primer for an amplification target circle (ATC). This allows rolling circle replication of an added ATC where the resulting tandem sequence DNA (TS-DNA) is coupled to the reporter molecule. Because of this, the TS-DNA will be effectively immobilized at the site of the target molecule. The sequence of the rolling circle replication primer sequence can be arbitrarily chosen. In a multiplex assay using multiple reporter molecules, it is preferred that the rolling circle replication primer sequence for each reporter molecule be substantially different to limit the possibility of non-specific target detection. When the reporter signal of a reporter molecule is used as a rolling circle replication primer, the reporter signal can be any length that supports specific and stable hybridization between the reporter signal and the primer complement portion of an amplification target circle. Generally this is 10 to 35 nucleotides long, but preferably is 16 to 20 nucleotides long.

In another embodiment, the reporter signal of a reporter molecule can include an amplification target circle which serves as a template for rolling circle replication. Replication of the ATC produces TS-DNA at the site where the reporter molecule is bound to the target molecule. For this purpose, the ATC should be tethered to the specific binding molecule by looping the ATC around a tether loop. This allows the ATC to rotate freely during rolling circle replication while remaining coupled to the affinity portion. The tether loop can be any material that can both form a loop and be coupled to a specific binding molecule. For example, linear polymers can be used for tether loops.

An amplification target circle (ATC) is a circular single-stranded DNA molecule, generally containing from 40 to 1,000 nucleotides, preferably from about 50 to 150 nucleotides, and most preferably from about 50 to 100 nucleotides. Portions of ATCs have specific functions making the ATC useful for rolling circle amplification (RCA). These portions are referred to as the primer complement portion and the reporter tag portions. The primer complement portion and the reporter tag portion are required elements of an amplification target circle. Those segments of the ATC that do not correspond to a specific portion of the ATC can be arbitrarily chosen sequences. It is preferred that ATCs do not have any sequences that are self-complementary. It is considered that this condition is met if there are no complementary regions greater than six nucleotides long without a mismatch or gap.

An amplification target circle, when replicated, gives rise to a long DNA molecule containing multiple repeats of sequences complementary to the amplification target circle. This long DNA molecule is referred to herein as tandem sequences DNA (TS-DNA). TS-DNA contains sequences complementary to the primer complement portion and the reporter tag portions. These sequences in the TS-DNA are referred to as primer sequences (which match the sequence of the rolling circle replication primer) and reporter tags. Amplification target circles and their use are further described in U.S. Patent No. 5,854,033.

Reporter Tags

Reporter tags are molecules or moieties that are produced during signal amplification of reporter molecules in the disclosed method and to which

decoding tags can associate. Reporter tags can be any type of molecule or moiety that can serve as a target for decoding tag association. Reporter tags are preferably the product of an amplification process, but need not be. For example, multiple reporter tags can be synthesized in any desired manner and associated en mass with a reporter molecule (thus effecting "amplification" of the reporter molecule's signal). Preferred reporter tags are oligomers, oligonucleotides, or nucleic acid sequences.

The oligomeric base sequences of oligomeric reporter tags can include RNA, DNA, modified RNA or DNA, modified backbone nucleotide-like oligomers such as peptide nucleic acid, methylphosphonate DNA, and 2'-O-methyl RNA or DNA. Oligomeric or oligonucleotide reporter tags can have any arbitrary sequence. The only requirement is association with decoding tags (preferably by hybridization). In the disclosed method, multiple reporter tags become associated with a single reporter signal (which is associated, via a specific binding molecule, to a target molecule). The context of these multiple reporter tags depends upon the technique used for signal amplification. Thus, where branched DNA is used, the branched DNA molecule includes the multiple reporter tags on the branches. Where oligonucleotide dendrimers are used, the reporter tags are on the dendrimer arms. Where rolling circle replication is used, multiple reporter tags result from the tandem repeats of complement of the amplification target circle sequence (which includes at least one complement of the reporter tag sequence). In this case, the reporter tags are tandemly repeated in the tandem sequence DNA.

Oligonucleotide reporter tags can each be any length that supports specific and stable hybridization between the reporter tags and the decoding tags. For this purpose, a length of 10 to 35 nucleotides is preferred, with a reporter tag 15 to 20 nucleotides long being most preferred.

The branched DNA for use in the disclosed method is generally known (Urdea, *Biotechnology* 12:926-928 (1994), and Horn *et al.*, *Nucleic Acids Res* 23:4835-4841 (1997)). As used herein, the tail of a branched DNA molecule refers to the portion of a branched DNA molecule that is designed to interact with the reporter signal. In general, each branched DNA molecule should have only one tail. The branches of the branched DNA (also referred to herein as the

arms of the branched DNA) contain reporter tag sequences. Oligonucleotide dendrimers (or dendrimeric DNA) are also generally known (Shchepinov et al., Nucleic Acids Res. 25:4447-4454 (1997), and Orentas et al., J. Virol. Methods 77:153-163 (1999)). As used herein, the tail of an oligonucleotide dendrimer refers to the portion of a dendrimer that is designed to interact with the reporter signal. In general, each dendrimer should have only one tail. The dendrimeric strands of the dendrimer are referred to herein as the arms of the oligonucleotide dendrimer and contain reporter tag sequences.

Decoding Tags

Decoding tags are any molecule or moiety that can be associated with reporter molecules, directly or indirectly, and which can be specifically detected. In particular, different decoding tags should be distinguishable upon detection. Decoding tags preferably are oligonucleotides, carbohydrates, synthetic polyamides, peptide nucleic acids, antibodies, ligands, proteins, haptens, zinc fingers, aptamers, or mass labels.

Preferred decoding tags are molecules capable of hybridizing specifically to an oligonucleotide reporter tag. Most preferred are peptide nucleic acid decoding tags. Oligonucleotide or peptide nucleic acid decoding tags can have any arbitrary sequence. The only requirement is hybridization to reporter tags. The decoding tags can each be any length that supports specific and stable hybridization between the reporter tags and the decoding tags. For this purpose, a length of 10 to 35 nucleotides is preferred, with a reporter tag 15 to 20 nucleotides long being most preferred.

Decoding tags can be detected using any suitable detection technique. Many molecular detection techniques are known and can be used in the disclosed method. For example, decoding tags can be detected by nuclear magnetic resonance, electron paramagnetic resonance, surface enhanced raman scattering, surface plasmon resonance, fluorescence, phosphorescence, chemiluminescence, resonance raman, microwave, mass spectrometry, or any combination of these. Decoding tags can be separated and/or detected by mass spectrometry, electrophoresis, or chromatography. Decoding tags can be distinguished temporally via different fluorescent, phosphorescent, or

chemiluminescent emission lifetimes. The composition and characteristics of decoding tags should be matched with the chosen detection method.

Decoding tags preferably are capable of being released by matrix-assisted laser desorption-ionization (MALDI) in order to be separated and identified (decoded) by time-of-flight (TOF) mass spectroscopy, or of being subjected to electrophoresis. A decoding tag may be any oligomeric molecule that can hybridize to a reporter tag. For example, a decoding tag can be a DNA oligonucleotide, an RNA oligonucleotide, or a peptide nucleic acid (PNA) molecule. The preferred decoding tags of this invention are PNA molecules.

For MALDI-TOF detection, the decoding tags preferably are peptide nucleic acids, where each decoding tag has a different mass to allow separation and separate detection in mass spectroscopy. For this purpose, it is preferable that each decoding tag have a similar number of nucleotide bases complementary to the reporter tag. This allows for more consistent hybridization characteristics while allowing the mass to vary. It is also preferable to use combination of base composition and number of mass tags (e.g. the number of 8-amino-3,6-dioxaoctanoic monomers attached to the PNA (Griffin, T.J., W. Tang, and L.M. Smith, *Genetic analysis by peptide nucleic acid affinity MALDI-TOF mass spectrometry.* Nat Biotechnol, 1997. **15**(12): p. 1368-72.)) to optimize the mass spectra for the set of decoding tags in a multiple tag analysis.

For capillary electrophoresis detection, the decoding tags preferably are fluorescently-labeled oligonucleotides, where each decoding tag has a different combination of length and fluorescent label. For this purpose, it is preferable that each decoding tag has the same number of nucleotides complementary to the reporter tag. It is also preferable that each decoding tag has a different number of nucleotides not complementary to the reporter tag. This allows for more consistent hybridization characteristics while allowing separation of the different decoding tags during electrophoresis.

Reporter Carriers

Reporter carriers are associations of one or more specific binding molecules, a carrier, and a plurality of decoding tags. Reporter carriers are used in the disclosed method to associate a large number of decoding tags with a

target molecule. The carrier can be any molecule or structure that facilitates association of many decoding tags with a specific binding molecule. Examples include liposomes, microparticles, nanoparticles, virons, phagmids, and branched polymer structures.

Selenium can substitute for sulfur in methionine, resulting in the modified amino acid selenomethionine. Selenium is approximately forty seven mass units larger than sulfur. Mass spectrometry may be used to identify peptides or proteins incorporating selenomethionine and methionine at a particular ratio. Small proteins and peptides with known selenium/sulfur ratio are preferably produced by chemical synthesis incorporating selenomethionine and methionine at the desired ratio. Larger proteins or peptides may be by produced from an *E. coli* expression system, or any other expression system that inserts selenomethionine and methionine at the desired ratio (Hendrickson, W.A., J.R. Horton, and D.M. LeMaster, *Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure.* Embo J, 1990. **9**(5): p. 1665-72, Cowie, D. and G. Cohen, *Biosynthesis by Escherichia coli of active altered proteins containing selenium instead of sulfur.* Biochimica et Biophysica Acta, 1957. **26**: p. 252 – 261, and Oikawa, T., *et al.*, *Metalloselenonein, the selenium analogue of metallothionein: synthesis and characterization of its complex with copper ions.* Proc Natl Acad Sci U S A, 1991. **88**(8): p. 3057-9.). Virion particles, or phagemids, or various capsid proteins, or assemblies, with these fixed selenium/sulfur ratio proteins or peptides, following attachment of a specific binding molecule, are examples of reporter carriers. The decoding tags (proteins or peptides) are preferably detected by mass spectrometry (e.g. Matrix Assisted Laser Desorption Ionization, MALDI, Mass Spectrometry or Secondary Ion Mass Spectrometry, SIMS).

A general class of carriers are structures and materials designed for drug delivery. Many such carriers are known. Liposomes are a preferred form of carrier.

Liposomes are artificial structures primarily composed of phospholipid bilayers. Cholesterol and fatty acids may also be included in the bilayer construction. Liposomes may be loaded with fluorescent tags, and coated on the

outer surface with specific recognition molecules (Truneh, A., Machy, P. and Horan, P.K., 1987, Antibody-bearing liposomes as multicolor immunofluorescent markers for flow cytometry and imaging. J. Immunol. Methods 100:59-71). However, the use of fluorescent liposomes in bioassays has been limited by the constraints of detection methods for fluorescent tags. Fluorescence-activated cell sorters typically have two or three different excitation-emission wavelengths, and microscopes typically have three or four excitation-emission filters. In some forms of the disclosed method, liposomes serve as carriers for arbitrary decoding tags. By combining liposome reporter carriers, loaded with arbitrary tags, with methods capable of separating a very large multiplicity of tags, it becomes possible to perform highly multiplexed assays.

Liposomes, preferably unilamellar vesicles, are made using established procedures that result in the loading of the interior compartment with a very large number (several thousand) of decoding tag molecules, where the chemical nature of these molecules is well suited for detection by a preselected detection method. Preferred detection methods and corresponding arbitrary tags are as follows: a) Mass spectrometry – oligopeptide tags; b) Electrophoresis – DNA oligonucleotide tags; c) Liquid chromatography – DNA tags or oligopeptide tags. Thus, one specific type of decoding tag preferably is used for each specific type of liposome-detector.

Each specific type of liposome reporter carrier is associated with a specific binding molecule. The association may be direct or indirect. An example of a direct association is a liposome containing covalently bound antibodies on the surface of the phospholipid bilayer. An alternative, indirect association composition is a liposome containing covalently bound DNA oligonucleotides of arbitrary sequence on its surface; these oligonucleotides are designed to recognize, by base complementarity, specific reporter molecules. The reporter molecule may comprise an antibody-DNA covalent complex, whereby the DNA portion of this complex can hybridize specifically with the complementary sequence on a liposome reporter carrier. In this fashion, the liposome reporter carrier becomes a generic reagent, which may be associated indirectly with any desired binding molecule.

The use of liposome reporter carriers can be illustrated with the following example.

1. Liposomes (preferably unilamellar vesicles with an average diameter of 150 to 300 nanometers) are prepared using the extrusion method (Hope, M.J., Bally, M.B., Webb, G., and Cullis, P.R., Biochimica et Biophysica Acta, 1985, 812:55-65; MacDonald, R.C., MacDonald, R.I., Menco, B., Takeshita, K., Subbarao, N., and Hu, L. Biochimica et Biophysica Acta, 1991, 1061:297-303). Other methods for liposome preparation may be used as well.

2. A solution of an oligopeptide, at a concentration 400 micromolar, is used during the preparation of the liposomes, such that the inner volume of the liposomes is loaded with this specific oligopeptide, which will serve to encode-decode the identity of a specific analyte of interest. A liposome with an internal diameter of 200 nanometers will contain, on the average, 960 molecules of the oligopeptide. Three separate preparations of liposomes are extruded, each loaded with a different oligopeptide. The oligopeptides are chosen such that their respective masses will be readily separable by MALDI-TOF mass spectrometry.

3. The outer surface of the three liposome preparations is conjugated with specific antibodies, as follows: a) the first liposome preparation is reacted with an antibody specific for the p53 tumor suppressor; b) the second liposome preparation is reacted with an antibody specific for the Bcl-2 oncoprotein; c) the third liposome preparation is reacted with an antibody specific or the Her2/neu membrane receptor. Coupling reactions are performed using standard procedures for the covalent coupling of antibodies to molecules. harboring reactive amino groups (Hendrickson, E.R., Hatfield, T.M., Joerger, R.D., Majarian, W.R., and Ebersole, R.C., 1995, Nucleic Acids Research, 23:522-529; Hermanson, G.T., Bioconjugate techniques, Academic Press, 1996, pp.528-569; Scheffold, A., Assenmacher, M., Reiners-Schramm, L., Lauster, R., and Radbruch, A., 2000, Nature Medicine 1:107-110). In the case of the liposomes, the reactive amino groups are those present in the phosphatidyl ethanolamine moieties of the liposomes.

4. A glass slide bearing a standard formaldehyde-fixed histological section is contacted with a mixture of all three liposome preparations, suspended

in a buffer containing 30 mM Tris-HCl, pH 7.6, 100 mM Sodium Chloride, 1 mM EDTA, 0.1 % Bovine serum albumin, in order to allow binding of the liposomes to the corresponding protein antigens present in the fixed tissue. After a one hour incubation, the slides are washed twice, for 5 minutes, with the same buffer (30 mM Tris-HCl, pH 7.6, 100 mM Sodium Chloride, 1 mM EDTA, 0.1 % Bovine serum albumin). The slides are dried with a stream of air.

5. The slides are coated with a thin layer of matrix solution consisting of 10 mg/ml alpha-cyano-4-hydroxycinnamic acid, 0.1% trifluoroacetic acid in a 50:50 mixture of acetonitrile in water. The slides are dried with a stream of air.

6. The slide is placed on the surface of a modified MALDI plate, an introduced in a Voyager DE Pro instrument (PerSeptive PE Biosystems, Framingham, MA). The machine is run in positive-ion reflector mode, with an ion extraction delay time of 250 ns.

7. Mass spectra are obtained from defined positions on the slide surface. The relative amount of each of the three peaks of encoding polypeptides is used to decode the relative ratios of the antigens detected by the liposome-detector complexes.

The liposome carrier method is not limited to the detection of analytes on histological sections. Cells obtained by sorting may also be used for analysis according to this invention (Scheffold, A., Assenmacher, M., Reiners-Schramm, L., Lauster, R., and Radbruch, A., 2000, Nature Medicine 1:107-110).

Target Samples

Any sample from any source can be used with the disclosed method. In general, target samples should be samples that contain, or may contain, target molecules. Examples of suitable target samples include cell samples, tissue samples, cell extracts, components or fractions purified from another sample, environmental samples, culture samples, tissue samples, bodily fluids, and biopsy samples. Numerous other sources of samples are known or can be developed and any can be used with the disclosed method. Preferred target samples for use with the disclosed method are samples of cells and tissues.

Target samples can be complex, simple, or anywhere in between. For example, a target sample may include a complex mixture of biological molecules (a tissue sample, for example), a target sample may be a highly

purified protein preparation, or a single type of molecule. Target molecules can be any molecule or portion of a molecule that is to be detected. Thus, a target molecule need not be a physically separate molecule, but may be a part of a larger molecule. Target molecules are also referred to as analytes.

Capture Arrays

A capture array (also referred to herein as an array) includes a plurality of capture tags immobilized at identified or predetermined locations on the array. In this context, plurality of capture tags refers to a multiple capture tags each having a different structure. Each predetermined location on the array (referred to herein as an array element) has one type of capture tag (that is, all the capture tags at that location have the same structure). Each location will have multiple copies of the capture tag. The spatial separation of capture tags of different structure in the array allows separate detection and identification of target molecules that become associated with the capture tags. If a decoding tag is detected at a given location in a capture array, it indicates that the target molecule corresponding to that array element was present in the target sample.

Reporter molecules and detector tags can also be immobilized in arrays. Different modes of the disclosed method can be performed with different components immobilized, labeled, or tagged. Arrays of reporter molecules and decoding tags can be made and used as described below and elsewhere herein for capture tags.

Solid-state substrates for use in capture arrays can include any solid material to which capture tags can be coupled, directly or indirectly. This includes materials such as acrylamide, cellulose, nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, glass, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Solid-state substrates can have any useful form including thin films or membranes, beads, bottles, dishes, fibers, woven fibers, shaped polymers, particles and microparticles. A preferred form for a solid-state substrate is a microtiter dish. The most preferred form of microtiter dish is the standard 96-well type.

Planar array technology has been utilized for many years (Shalon, D., S.J. Smith, and P.O. Brown, *A DNA microarray system for analyzing complex DNA samples using two- color fluorescent probe hybridization.* Genome Res, 1996. **6**(7): p. 639-45, Singh-Gasson, S., *et al.*, *Maskless fabrication of light-directed oligonucleotide microarrays using a digital micromirror array.* Nat Biotechnol, 1999. **17**(10): p. 974-8, Southern, E.M., U. Maskos, and J.K. Elder, *Analyzing and comparing nucleic acid sequences by hybridization to arrays of oligonucleotides: evaluation using experimental models.* Genomics, 1992. **13**(4): p. 1008-17, Nizetic, D., *et al.*, *Construction, arraying, and high-density screening of large insert libraries of human chromosomes X and 21: their potential use as reference libraries.* Proc Natl Acad Sci U S A, 1991. **88**(8): p. 3233-7, Van Oss, C.J., R.J. Good, and M.K. Chaudhury, *Mechanism of DNA (Southern) and protein (Western) blotting on cellulose nitrate and other membranes.* J Chromatogr, 1987. **391**(1): p. 53-65, Ramsay, G., *DNA chips: state-of-the art.* Nat Biotechnol, 1998. **16**(1): p. 40-4, Schena, M., *et al.*, *Parallel human genome analysis: microarray-based expression monitoring of 1000 genes.* Proc Natl Acad Sci U S A, 1996. **93**(20): p. 10614-9, Lipshutz, R.J., *et al.*, *High density synthetic oligonucleotide arrays.* Nat Genet, 1999. **21**(1 Suppl): p. 20-4, Pease, A.C., *et al.*, *Light-generated oligonucleotide arrays for rapid DNA sequence analysis.* Proc Natl Acad Sci U S A, 1994. **91**(11): p. 5022-6, Maier, E., *et al.*, *Application of robotic technology to automated sequence fingerprint analysis by oligonucleotide hybridisation.* J Biotechnol, 1994. **35**(2-3): p. 191-203, Vasiliskov, A.V., *et al.*, *Fabrication of microarray of gel-immobilized compounds on a chip by copolymerization.* Biotechniques, 1999. **27**(3): p. 592-4, 596-8, 600 passim, and Yershov, G., *et al.*, *DNA analysis and diagnostics on oligonucleotide microchips.* Proc Natl Acad Sci U S A, 1996. **93**(10): p. 4913-8). Such arrays may be constructed upon non permeable or permeable supports of a wide variety of support composition, for example nylon, cellulose, glass, polymer on glass, and many others. The array spot sizes and density of spot packing vary over a tremendous range depending upon the process(es) and material(s) used.

Methods for immobilization of oligonucleotides to solid-state substrates are well established. Oligonucleotide capture tags can be coupled to substrates

using established coupling methods. For example, suitable attachment methods are described by Pease *et al., Proc. Natl. Acad. Sci. USA* **91**(11):5022-5026 (1994), and Khrapko *et al., Mol Biol (Mosk) (USSR)* 25:718-730 (1991). A method for immobilization of 3'-amine oligonucleotides on casein-coated slides is described by Stimpson *et al., Proc. Natl. Acad. Sci. USA* **92**:6379-6383 (1995). A preferred method of attaching oligonucleotides to solid-state substrates is described by Guo *et al., Nucleic Acids Res.* 22:5456-5465 (1994).

Methods for producing arrays of oligonucleotides on solid-state substrates are also known. Examples of such techniques are described in U.S. Patent No. 5,871,928 to Fodor et al., U.S. Patent No. 5,654,413 to Brenner, U.S. Patent No. 5,429,807, and U.S. Patent No. 5,599,695 to Pease et al.

Although preferred, it is not required that a given capture array be a single unit or structure. The set of capture tags may be distributed over any number of solid supports. For example, at one extreme, each capture tag may be immobilized in a separate reaction tube or container.

Oligonucleotide capture tags in arrays can also be designed to have similar hybrid stability. This would make hybridization of fragments to such capture tags more efficient and reduce the incidence of mismatch hybridization. The hybrid stability of oligonucleotide capture tags can be calculated using known formulas and principles of thermodynamics (see, for example, Santa Lucia et al., *Biochemistry* 35:3555-3562 (1996); Freier et al., *Proc. Natl. Acad. Sci. USA* 83:9373-9377 (1986); Breslauer et al., *Proc. Natl. Acad. Sci. USA* 83:3746-3750 (1986)). The hybrid stability of the oligonucleotide capture tags can be made more similar (a process that can be referred to as smoothing the hybrid stabilities) by, for example, chemically modifying the capture tags (Nguyen et al., *Nucleic Acids Res.* 25(15):3059-3065 (1997); Hohsisel, *Nucleic Acids Res.* 24(3):430-432 (1996)). Hybrid stability can also be smoothed by carrying out the hybridization under specialized conditions (Nguyen et al., *Nucleic Acids Res.* 27(6):1492-1498 (1999); Wood et al., *Proc. Natl. Acad. Sci.* USA 82(6):1585-1588 (1985)).

Another means of smoothing hybrid stability of the oligonucleotide capture tags is to vary the length of the capture tags. This would allow adjustment of the hybrid stability of each capture tag so that all of the capture

tags had similar hybrid stabilities (to the extent possible). Since the addition or deletion of a single nucleotide from a capture tag will change the hybrid stability of the capture tag by a fixed increment, it is understood that the hybrid stabilities of the capture tags in a capture array will not be equal. For this reason, similarity of hybrid stability as used herein refers to any increase in the similarity of the hybrid stabilities of the capture tags (or, put another way, any reduction in the differences in hybrid stabilities of the capture tags).

The efficiency of hybridization and ligation of oligonucleotide capture tags to sample fragments can also be improved by grouping capture tags of similar hybrid stability in sections or segments of a capture array that can be subjected to different hybridization conditions. In this way, the hybridization conditions can be optimized for particular classes of capture tags.

Capture Tags

A capture tag is any compound that can be used to capture or separate compounds or complexes having the capture tag. Preferably, a capture tag is a compound, such as a ligand or hapten, that binds to or interacts with another compound, such as ligand-binding molecule or an antibody. It is also preferred that such interaction between the capture tag and the capturing component be a specific interaction, such as between a hapten and an antibody or a ligand and a ligand-binding molecule.

Preferred capture tags, described in the context of nucleic acid probes, are described by Syvnen *et al., Nucleic Acids Res.*, 14:5037 (1986). Preferred capture tags include biotin, which can be incorporated into nucleic acids. Capturing sample fragments on a substrate may be accomplished in several ways. In one embodiment, capture docks are adhered or coupled to the substrate. Capture docks are compounds or moieties that mediate adherence of a sample fragment by binding to, or interacting with, a capture tag on the fragment. Capture docks immobilized on a substrate allow capture of the fragment on the substrate. Such capture provides a convenient means of washing away reaction components that might interfere with subsequent steps.

Substrates for use in the disclosed method can include any solid material to which components of the assay can be adhered or coupled. Examples of substrates include, but are not limited to, materials such as acrylamide, cellulose,

nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Substrates can have any useful form including thin films or membranes, beads, bottles, dishes, fibers, woven fibers, shaped polymers, particles and microparticles. Preferred forms of substrates are plates and beads. The most preferred form of beads are magnetic beads.

In one embodiment, capture tags and capture docks can be oligonucleotides. Methods for immobilizing and coupling oligonucleotides to substrates are well established. For example, suitable attachment methods are described by Pease *et al.*, *Proc. Natl. Acad. Sci. USA* 91(11):5022-5026 (1994), and Khrapko *et al.*, *Mol Biol (Mosk) (USSR)* 25:718-730 (1991). A method for immobilization of 3'-amine oligonucleotides on casein-coated slides is described by Stimpson *et al.*, *Proc. Natl. Acad. Sci. USA* 92:6379-6383 (1995). A preferred method of attaching oligonucleotides to solid-state substrates is described by Guo *et al.*, *Nucleic Acids Res.* 22:5456-5465 (1994).

In another embodiment, caputre tags and capture docks can be anti-hybrid antibodies. Methods for immobilizing antibodies to substrates are well established. Immobilization can be accomplished by attachment, for example, to aminated surfaces, carboxylated surfaces or hydroxylated surfaces using standard immobilization chemistries. Examples of attachment agents are cyanogen bromide, succinimide, aldehydes, tosyl chloride, avidin-biotin, photocrosslinkable agents, epoxides and maleimides. A preferred attachment agent is glutaraldehyde. These and other attachment agents, as well as methods for their use in attachment, are described in *Protein immobilization: fundamentals and applications*, Richard F. Taylor, ed. (M. Dekker, New York, 1991), Johnstone and Thorpe, *Immunochemistry In Practice* (Blackwell Scientific Publications, Oxford, England, 1987) pages 209-216 and 241-242, and *Immobilized Affinity Ligands*, Craig T. Hermanson *et al.*, eds. (Academic Press, New York, 1992). Antibodies can be attached to a substrate by chemically cross-linking a free amino group on the antibody to reactive side groups present

within the substrate. For example, antibodies may be chemically cross-linked to a substrate that contains free amino or carboxyl groups using glutaraldehyde or carbodiimides as cross-linker agents. In this method, aqueous solutions containing free antibodies are incubated with the solid-state substrate in the presence of glutaraldehyde or carbodiimide. For crosslinking with glutaraldehyde the reactants can be incubated with 2% glutaraldehyde by volume in a buffered solution such as 0.1 M sodium cacodylate at pH 7.4. Other standard immobilization chemistries are known by those of skill in the art.

Sorting Tags

A sorting tag is any compound that can be used to sort or separate compounds or complexes having the sorting tag from those that do not. In general, all capture tags can be a sorting tag. Sorting tags also include compounds and moieties that can be detected and which can mediate the sorting of tagged components. Such forms of sorting tags are generally not also capture tags. For example, a fluorescent moiety can allow sorting of components tagged with the moiety from those that are not (or those with a different tag). However, such a fluorescent moiety does not necessarily have a suitable capture dock with which it can interact and be captured. Preferably, a sorting tag is a label, such as a fluorescent label, that can mediate sorting.

Method

The disclosed method is based on encoding target molecules with signals followed by decoding of the encoded signal. This encoding/decoding uncouples the detection of a target molecule from the chemical and physical properties of the target molecule. In basic form, the disclosed method involves association of one or more reporter molecules with one or more target samples, association of one or more decoding tags with the reporter molecules, and detection of the decoding tags. The reporter molecules associate with target molecules in the target sample(s). Generally, the reporter molecules correspond to one or more target molecules, and the decoding tags correspond to one or more reporter molecules. Thus, detection of particular decoding tags indicates the presence of the corresponding reporter molecules. In turn, the presence of particular reporter molecules indicates the presence of the corresponding target molecules.

This indirect detection uncouples the detection of target molecules from the chemical and physical properties of the target molecules by interposing decoding tags that essentially can have any arbitrary chemical and physical properties. In particular, decoding tags can have specific properties useful for detection, and decoding tags within an assay can have highly ordered or structured relationships with each other. It is the (freely chosen) properties of the decoding tags, rather than the (take them as they are) properties of the target molecules that matters at the point of detection.

The decoding tags have the additional advantage of being uncoupled from the target molecule-specific aspects of the reporter molecules. Unlike detection methods where a labeled molecule is bound to an analyte followed by detection of the label, the disclosed method is not limited by the chemical and physical properties of the labeled molecule. This allows more convenient detection, more sensitive detection, and more highly multiplexed detection schemes.

The sensitivity of the disclosed method can also be enhanced by including a signal amplification step prior to detection. In basic form, amplification is accomplished by amplifying reporter signals on the reporter molecules. This results in multiple reporter tags associated with each reporter molecule. The decoding tags are then associated with the reporter tags and detected. Generally, the decoding tags correspond to one or more reporter tags (and thus correspond to the reporter molecules with which the reporter tags are associated), and the reporter tags correspond to the reporter molecules with which they are associated. Thus, detection of particular decoding tags indicates the presence of the corresponding reporter tags. In turn, the presence of particular reporter tags indicates the presence of the corresponding reporter molecules. In turn, the presence of particular reporter molecules indicates the presence of the corresponding target molecules. The reporter molecule also can be designed to include multiple reporter tags (essentially accomplishing a pre-assay amplification of the signal).

In general, the target sample is expected to contain, or is suspected of containing, a plurality of different target molecules. The specific binding molecule of each reporter molecule is selected to interact with one of the target

molecules. A set of specific binding molecules can be selected to interact with all of the target molecules of interest in the sample. The target sample preferably is immobilized, fixed, or adhered to a surface. Alternatively, reporter molecules can be associated with a sample, or the source of a sample, prior to immobilization, fixation, or adherence. Either case allows the locations of the target molecules on the surface to be determined in the method by associating decoding tags with the target molecules and detecting the location of the decoding tags.

Any number of reporter molecules can be used when performing the disclosed method. The disclosed method is especially useful for detection of multiple target molecules in a single assay. This multiplexing is made convenient in the disclosed method by the uncoupling of the chemical nature of the target molecules and the chemical nature of the decoding tags, and multiple codings that can be embodied in the decoding tags. Accordingly, it is preferred that four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, twenty or more, forty or more, eighty or more, or one hundred or more reporter molecules be used in a single performance of the disclosed method. The number of reporter molecules that can be used are not limited to these ranges but can include any sub-range. For example, thirty or more, fifty-five or more, seventy-two or more, and so on, reporter molecules can be used. All of the included sub-ranges are specifically contemplated.

Multiple Tag Analysis

In one form of the disclosed method, one or more target samples and one or more reporter molecules are brought into contact, allowing the reporter molecules to become associated with target molecules in the target samples. The reporter molecules are then amplified to produce multiple reporter tags for each reporter molecule. The reporter tags remain associated with the reporter molecules. One or more decoding tags are then associated with the reporter tags, and the decoding tags are detected. The decoding tags correspond to reporter molecules and the reporter molecules correspond to target molecules. This relationship means that detecting the decoding tags indicates the presence of reporter molecules corresponding to the detected decoding tags, and that the

presence of reporter molecules indicates the presence of target molecules corresponding to the reporter molecules.

This form of the disclosed method is based on specific recognition of analytes in a sample, amplification of the recognition event, and detection of the amplified signal. The specific recognition is accomplished with specific binding molecules that bind or otherwise interact specifically with an analyte of interest. The amplification of this interaction is mediated by a reporter signal coupled, tethered, or otherwise attached to the specific binding molecule. It is the reporter signal, an oligonucleotide, that is used to produce multiple reporter tags that remain associated with the reporter signal (and thus also with the specific binding molecule and analyte). This is accomplished, for example, by amplification of the reporter signal or hybridization of branched DNA or oligonucleotide dendrimers to the reporter signals. Detector tags, which are coded to include distinct, separately detectable attributes, are then hybridized to the reporter tags. Detection of the various detector tags results in indirect detection of the various analytes in the sample.

A key to the method is the use of distinct reporter signals for each different analyte, the subsequent association of distinct reporter tags with the various reporter signals, and the subsequent hybridization of distinct decoding tags to the various reporter tags. In this way, each different type of analyte ends up with a different and separately detectable decoding tag associated with it. The combination of specific binding molecule and reporter signal results in an effective conversion of the different analytes (where the analytes may be widely chemically divergent) into a standardized signal (the reporter signal). These standardized signals (which are coded for each analyte) are then amplified and detected using a single set of standardized conditions (during which the coding is carried forward). The detection of the coded, amplified signals (that is, the decoding tags) results in effective, convenient detection of multiple analytes in a single assay.

An example of this form of the disclosed method has the following basic steps:

(a) bringing into contact one or more target samples and one or more reporter molecules, where each reporter molecule includes a reporter signal and

a specific binding molecule. The reporter signal preferably is an oligonucleotide coupled or tethered to the specific binding molecule. The specific binding molecule of each reporter molecule is chosen to interact with a different target molecule.

(b) amplifying the reporter signal to produce multiple reporter tags for each reporter signal, where the reporter tags remain associated with the reporter signal. The reporter tags produced from the reporter signal of each reporter molecule preferably are different from the reporter tags produced from the reporter signals of other reporter molecules.

(c) associating decoding tags to the reporter tags, where a different decoding tag corresponds to each different reporter tag.

(d) detecting the decoding tags associated with the reporter tags. The detected decoding tags are indicative of the location and amount of target molecules in the target sample.

Multiple Tag Analysis of Multiple Samples

In another form of the disclosed method, four or more target samples and one or more reporter molecules are brought into contact, allowing the reporter molecules to become associated with target molecules in the target samples. Each target sample is brought into contact with a different set of reporter molecules, and the reporter molecules in each set of reporter molecules are different from the reporter molecules in the other sets of reporter molecules. The four or more of the target samples are then mixed together, one or more decoding tags are associated with the reporter molecules, and the decoding tags are detected. A different decoding tag corresponds to each different reporter molecule such that each decoding tag corresponds to only one of the target samples. This relationship means that detecting the decoding tags indicates the presence of target molecules corresponding to the detected decoding tags. Further, detection of decoding tags corresponding to different target samples indicates the presence of the same target molecules in the corresponding target samples.

In this form of the method, it is preferred that the reporter molecules be associated with target molecules by covalently coupling the reporter molecules to the target molecules. For example, the reporter molecules can be associated

with target molecules by incorporating the reporter molecules during synthesis of the target molecules, or by reacting a reactive group on the reporter molecules with the target molecules. A preferred way to incorporate reporter molecules into target molecules is to include a primer portion in the reporter molecule that is used to prime synthesis of a nucleic acid molecule.

An example of this form of the disclosed method has the following basic steps:

(a) bringing into contact four or more target samples and one or more reporter molecules, where each target sample is brought into contact with a different set of reporter molecules. The reporter molecules in each set of reporter molecules are different from the reporter molecules in the other sets of reporter molecules, and the reporter molecules are associated with target molecules in the target samples.

(b) mixing together four or more of the target samples.

(c) associating one or more decoding tags with the reporter molecules, where a different decoding tag corresponds to each different reporter molecule such that each decoding tag corresponds to only one of the target samples.

(d) detecting the decoding tags. The decoding tags are disassociated from the reporter molecules during, or prior to, detection. Detection of the decoding tags indicates the presence of target molecules corresponding to the detected decoding tags. Detection of decoding tags corresponding to different target samples indicates the presence of the same target molecules in the corresponding target samples.

This form of the method can also include, prior to step (c), amplifying the reporter molecules to produce multiple reporter tags for each reporter molecule. The reporter tags remain associated with the reporter molecule. The reporter tags produced from each reporter molecule are different from the reporter tags produced from other reporter molecules, and the decoding tags are associated with the reporter molecules by associating the decoding tags with the reporter tags.

In preferred embodiments of this form of the method, each reporter molecule can comprise a reporter signal such that each reporter molecule in the same set of reporter molecules has the same reporter signal. Amplification of

the reporter molecules is then accomplished by amplifying the reporter signals. A plurality of decoding tags can correspond to each target sample.

The reporter molecules can be associated with target molecules by covalently coupling the reporter molecules to the target molecules. For example, the reporter molecules can be associated with target molecules by incorporating the reporter molecules during synthesis of the target molecules. Alternatively, the reporter molecules can be covalently coupled to the target molecules by reacting a reactive group on the reporter molecules with the target molecules. Each reporter molecule can comprise a primer portion and a reporter signal, where the primer portions prime synthesis of the target molecules. The decoding tags can then be associated with the reporter molecules by associating the decoding tags with the reporter signals. The reporter signals can be oligonucleotides, and the decoding tags can be peptide nucleic acids that are complementary to the reporter signals.

This form of the method is useful for comparing a large number of experimental samples. In a typical case, a large batch of experimental animals is treated with a drug, and specific organs, such as brain, liver, kidney, bladder, lung, and ovary or testis are harvested for analysis. Such an experiment may involve 24 experimental animals, where 6 different tissues are harvested at 4 time points. Using prior technology, one must then perform 24 microarray experiments, where mRNA or protein is extracted from each experimental organ-time point is compared to a control mRNA or protein from the same organ or tissue. These 24 experiments consume considerable time and resources (including the significant cost of the 24 microarrays).

One embodiment of the disclosed method allows a multiplexed, or parallel analysis of an entire panel of mRNA samples in a single microarray experiment. Another embodiment of the disclosed method allows a multiplexed, or parallel analysis of an entire panel of protein samples in a single microarray experiment. Examples of both embodiments are described below.

In the first, an experimental animal is treated with a drug. To study toxicity effects, messenger RNA is extracted from six different tissue biopsies of the experimental animal at time zero (just before drug administration), and at

another three time points (days 1, 2, 3). A total of 24 different mRNA preparations is obtained.

The messenger RNA is labeled as follows: A first strand of cDNA is generated using reverse transcriptase, and the RNA strand is destroyed with alkali. After ethanol precipitation, the RNA is copied by random priming using a random octamer (the specific binding molecule) tethered at its 5'-end to an arbitrary DNA zip sequence of 20 bases (the reporter signal), the zip sequence preferably comprising 50% isoG and isoC residues (Collins ML, Irvine B, Tyner D, Fine E, Zayati C, Chang C, Horn T, Ahle D, Detmer J, Shen LP, Kolberg J, Bushnell S, Urdea MS, Ho DD (1997) A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. *Nucleic Acids Res* 25:2979-2984). For each of the 24 different mRNA preparations, a unique random octamer-zip sequence is used, designed according to the rules described by (Gerry NP, Witowski NE, Day J, Hammer RP, Barany G, Barany F (1999) Universal DNA microarray method for multiplex detection of low abundance point mutations. *J Mol Biol* 292:251-62).

All of the 24 random-primed cDNA preparations, each harboring unique zip sequences, are pooled together before being used for hybridization. The pooled probes are hybridized to a cDNA microarray made on glass slides. After overnight hybridization, the slides are washed using standard procedures, and are then hybridized with 24 different branch DNA (bDNA) amplifier assemblies (Hendricks DA, Stowe BJ, Hoo BS, Kolberg J, Irvine BD, Neuwald PD, Urdea MS, Perrillo RP (1995) Quantitation of HBV DNA in human serum using a branched DNA (bDNA) signal amplification assay. *Am J Clin Pathol* 104:537-46) tethered to isoG and isoC containing anti-zip oligos complementary to each of the 24 random primer zip oligonucleotides. After washing excess bDNA, the slides are hybridized with 24 PNA mass tags (these are the decoding tags), each capable of hybridizing to a single specific bDNA amplified tag sequence, and each having a different molecular weight.

After washing, the microarrays are covered with a matrix solution, dried, and placed in a mass spectrometer. The laser beam is directed sequentially to each of the microarray dots and MALDI-TOF mass spectra are generated for all dots.

The intensity of each peak, corresponding to the different PNA mass tags, is recorded, in order to measure the expression of the mRNA corresponding to each tag in each tissue time point (for a total of 24 data points per spot on the microarray).

In the above example, which involved mRNA profiling, 6 different tissues are assayed at 4 different time points, from a total of 24 experimental animals. Likewise, protein may be extracted from each sample. Each protein preparation can be tagged by covalent coupling of a DNA oligonucleotide. All 24 protein preparations are then mixed together, and contacted with the antibody microarray. After washing unbound proteins, the bDNA oligos are amplified, and PNA decoding tags are bound. Signal readout is performed by mass spectrometry (electrophoresis and HPLC are less preferred options). This experiment will yield data on the relative expression level of each protein for which there exists a cognate antibody on the microarray. Relative protein expression levels are obtained for all of the 24 experimental time points.

In the second example, an experimental animal is treated with a drug. To study toxicity effects, protein is extracted from six different tissue biopsies of the experimental animal at time zero (just before drug administration), and at another three time points (days 1, 2, 3). A total of 24 different protein preparations is obtained.

Each of the protein preparations is tagged with a unique DNA oligonucleotide as follows:

The protein preparation is reacted with 2-iminothiolane to introduce reactive sulfhydryl groups, if none is present. A DNA oligonucleotide, containing a reactive amino group at one of its termini is reacted with a heterobifunctional cross-linking reagent, such as SULFO-SMCC (Pierce, Inc.). The thiol-containing proteins are incubated together with the activated oligonucleotide, to form a covalent protein-DNA adduct. For most protein molecules, the formation of this covalent adduct will not interfere with the capacity of the protein to bind to its cognate antibody.

All 24 protein preparations, each harboring covalently bound unique DNA tag sequences, are pooled together before being used for an antibody microarray experiment. The pooled proteins are contacted with a microarray

made by spotting tens, hundreds, or thousands of specific antibodies at different locations on glass slides (Ekins and Chu, 1991). After overnight incubation, the slides are washed using standard immunoassay procedures, and are then hybridized with 24 different branch DNA (bDNA) amplifier assemblies (Hendricks et al., 1995).

After washing away excess bDNA, the slides are hybridized with 24 different PNA mass tags, each capable of hybridizing to a single specific bDNA amplified tag sequence, and each having a unique molecular weight. After washing, the microarrays are covered with a matrix solution, dried, and placed in a mass spectrometer. The laser beam is directed sequentially to each of the microarray dots and MALDI-TOF mass spectra are generated for all dots. The intensity of each peak, corresponding to the 24 different PNA mass tags, is recorded at each dot on the microarray, in order to measure the relative amounts of each of the tagged proteins (each bound to its specific, cognate antibody on the surface of the microarray) from each of the two experimental samples.

Multiple Tag Analysis Without Amplification

The decoding tags of the disclosed method have the advantage of being uncoupled from the target molecule-specific aspects of the reporter molecules. Unlike detection methods where a labeled molecule is bound to an analyte followed by detection of the label, the disclosed method is not limited by the chemical and physical properties of the labeled molecule. This allows more convenient detection, more sensitive detection, and more highly multiplexed detection schemes.

In another form of the disclosed method, one or more target samples and one or more reporter molecules are brought into contact, allowing the reporter molecules to become associated with target molecules in the target samples. One or more decoding tags are then associated with the reporter molecules. A different decoding tag corresponds to each different reporter molecule, and the decoding tags are not covalently coupled to the reporter molecules. The decoding tags are then detected by disassociating the decoding tags from the reporter molecules. The decoding tags correspond to reporter molecules, and the reporter molecules correspond to target molecules. This relationship means that detection of the decoding tags indicates the presence of reporter molecules

corresponding to the detected decoding tags, and that the presence of reporter molecules indicates the presence of target molecules corresponding to the reporter molecules.

An example of this form of the disclosed method has the following basic steps:

(a) bringing into contact one or more target samples and one or more reporter molecules, where the reporter molecules are associated with target molecules in the target samples.

(b) associating one or more decoding tags with the reporter molecules. A different decoding tag corresponds to each different reporter molecule. The decoding tags are not covalently coupled to the reporter molecules.

(c) detecting the decoding tags. The decoding tags are disassociated from the reporter molecules during, or prior to, detection. The decoding tags correspond to reporter molecules. The reporter molecules correspond to target molecules. Detection of the decoding tags indicates the presence of reporter molecules corresponding to the detected decoding tags, and the presence of reporter molecules indicates the presence of target molecules corresponding to the reporter molecules.

In preferred embodiments of this form of the method, each reporter molecule can comprise one or more specific binding molecules such that the specific binding molecule of each reporter molecule interacts with a different target molecule.

This form of the method can also include, prior to step (c), amplifying the reporter molecules to produce multiple reporter tags for each reporter molecule. The reporter tags remain associated with the reporter molecule. The reporter tags produced from each reporter molecule are different from the reporter tags produced from other reporter molecules, and the decoding tags are associated with the reporter molecules by associating the decoding tags with the reporter tags.

As an example of this form of the method, consider a nucleic acid construct (reporter molecule) that contains two domains, a detection domain (specific binding molecule) and an encoding domain (reporter signal),

5'-<u>GCATCGCATCGGATCGATCG</u>ACGGGGCAGA-3'

The underlined sequence, 5'-<u>GCATCGCATCGGATCGATCG</u>-3', represents specific binding molecule of a reporter molecule that will specifically hybridize to a cognate single stranded nucleic acid target molecule locus of interest. The remaining part of this construct, 5'-ACGGGGCAGA-3', represents the reporter signal of a reporter molecule. A PNA that is complementary to all or part of the reporter signal sequence may act as the decoding tag. The binding of PNA:DNA duplexes is stronger than the binding of DNA:DNA duplexes (Chakrabarti, M.C. and F.P. Schwarz, *Thermal stability of PNA/DNA and DNA/DNA duplexes by differential scanning calorimetry.* Nucleic Acids Res, 1999. **27**(24): p. 4801-6; Schwarz, F.P., S. Robinson, and J.M. Butler, *Thermodynamic comparison of PNA/DNA and DNA/DNA hybridization reactions at ambient temperature.* Nucleic Acids Res, 1999. **27**(24): p. 4792-800), thus the PNA will remain bound to this construct in the presence of a DNA strand of the same sequence. Use of isoC and isoG (Collins, M.L., *et al.*, *A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml.* Nucleic Acids Res, 1997. **25**(15): p. 2979-84; Switzer, C.Y., S.E. Moroney, and S.A. Benner, *Enzymatic recognition of the base pair between isocytidine and isoguanosine.* Biochemistry, 1993. **32**(39): p. 10489-96; Horn, T., C.A. Chang, and M.L. Collins, Tetrahedron Lett., 1995. **36**: p. 2033-2036) in the reporter signal and the decoding tag will make these sequences unable to hybridize with the target sequence.

As an illustration of the use of this example method, consider the following.

The construct is produced by mixing the single stranded nucleic acid with the cognate decoding PNA. Within a set, such constructs are produced individually. The DNA target molecule of interest is amplified by PCR with modified primers. The primers are designed such that the resultant DNA strand will contain a 3' biotin moiety, and the 5' end contains at least four phosphorothioate linked nucleotides rather than phosphodiester linkages.

The double stranded PCR amplicons are digested by T7 gene 6 to yield single stranded DNA (Nikiforov, T.T., *et al.*, *The use of phosphorothioate primers and exonuclease hydrolysis for the preparation of single-stranded PCR*

products and their detection by solid-phase hybridization. PCR Methods Appl, 1994. **3**(5): p. 285-91). The set of constructs is contacted with the sample under conditions for hybridization of the probe sequence with the cognate DNA sample. The mixture is transferred to an avidin coated well where the PCR amplicons are captured by the biotin-avidin interaction and the excess and mismatched constructs are washed away. A low salt wash is performed to release from the DNA target molecules the reporter molecules with their associated decoding tags. The liquid phase is transferred to a well of an autosampler for detection of the multiple PNA decoding tags.

Each PNA decoding tag sequence is detected by its unique mass spectrum, which uniquely identifies the decoding tag. The detector is, for example, an Electrospray Time-of-Flight Mass Spectrometer. In the mass spectrometer source the oligonucleotide is dissociated from the PNA decoding sequence, and the PNA is detected. Alternately, the decoding sequence can be a chromatographically or electrophoretically separable moiety. Use of any of these techniques for multiple tag analysis enables the detection and identification of hundreds, or even thousands, of different decoding tags, which in turn identifies hundreds, or even thousands, of different target molecules.

A preferred construct would utilize an improvement of a molecular switch (Lizardi, P.M., *et al.*, *Nucleic acid process containing improved molecular switch*, 1992.United States Patent 5,118,801). Related probe molecules labeled with a fluorophore at one terminus and a quencher at the other terminus have been called molecular beacons (Tyagi, S., D.P. Bratu, and F.R. Kramer, *Multicolor molecular beacons for allele discrimination.* Nat Biotechnol, 1998. **16**(1): p. 49-53; Tyagi, S. and F.R. Kramer, *Molecular beacons: probes that fluoresce upon hybridization.* Nat Biotechnol, 1996. **14**(3): p. 303-8; Marras, S.A., F.R. Kramer, and S. Tyagi, *Multiplex detection of single-nucleotide variations using molecular beacons.* Genet Anal, 1999. **14**(5-6): p. 151-6; Vet, J.A., *et al.*, *Multiplex detection of four pathogenic retroviruses using molecular beacons.* Proc Natl Acad Sci U S A, 1999. **96**(11): p. 6394-9; Manganelli, R., *et al.*, *Differential expression of 10 sigma factor genes in Mycobacterium tuberculosis.* Mol Microbiol, 1999. **31**(2): p. 715-24) and have been demonstrated to have better detection specificity than their linear

equivalent probe sequence (Bonnet, G., *et al.*, *Thermodynamic basis of the enhanced specificity of structured DNA probes.* Proc Natl Acad Sci U S A, 1999. **96**(11): p. 6171-6). The beacons are limited in the extent of multiplexing due to spectral overlap of the readout fluorophores. In the current invention, the specificity of the molecular switch is exploited, and a much higher multiplex readout is enabled. An example of such a construct is:

5'-XM<u>GCATCGCATCGGATCGATCG</u>NYACGGGGCAGA-3'

where M and N indicate a (typically small) number of bases; X and Y are short sequences that are complementary to each other, typically 5 to 8 bases; and, detection and encoding domains as indicated above. Under the hybridization conditions this construct will form a 'step and loop' structure (where X and Y are hybridized to each other) which competes with the probe sequence hybridizing to the target sequence.

Multiple Tag Analysis With Reporter Carriers

In another form of the disclosed method, one or more target samples and one or more reporter carriers are brought into contact. The reporter carriers include one or more specific binding molecules, a carrier, and a plurality of decoding tags associated with the carrier. After the reporter carriers are associated with reporter molecules, the decoding tags are detected. The decoding tags correspond to reporter carriers, and the reporter carriers correspond to target molecules. This relationship means that detection of the decoding tags indicates the presence of reporter carriers corresponding to the detected decoding tags, and that the presence of reporter carriers indicates the presence of target molecules corresponding to the reporter carriers.

An example of this form of the disclosed method has the following basic steps:

(a) bringing into contact one or more target samples and one or more reporter carriers, where each reporter carrier comprises one or more specific binding molecules, a carrier, and a plurality of decoding tags associated with the carrier.

(b) detecting the decoding tags. The decoding tags correspond to reporter carriers and the reporter carriers correspond to target molecules. Detecting the decoding tags indicates the presence of reporter carriers

corresponding to the detected decoding tags, and the presence of reporter carriers indicates the presence of target molecules corresponding to the reporter carriers.

The carrier can be a liposome. Each reporter carrier preferably includes at least 1,000 decoding tags. The decoding tags preferably are detected by mass spectrometry, electrophoresis, or chromatography. The carrier can also be a viral particle, wherein the decoding tags are viral proteins containing selenium-substituted methionine, where the decoding tags are detected by mass spectrometry such that different decoding tags are distinguished by selenium-based differences in mass.

The specific binding molecules of each reporter carrier can interact with a different target molecule such that a different decoding tag corresponds to each different reporter carrier. The decoding tags can be disassociated from the reporter carriers during, or prior to, detection.

Other Features

All forms of the disclosed method can be performed using a variety of specific components or additional steps. For example, the reporter molecules can be amplified using branched DNA. For this it is preferred that each reporter molecule comprise a reporter signal and each branched DNA comprise multiple reporter tags and a tail which associates with the reporter signal.

The reporter molecules can also be amplified using rolling circle amplification. For this it is preferred that each reporter molecule comprises a reporter signal, where the reporter signal primes rolling circle replication of an amplification target circle and the amplification target circle comprises one or more sequences complementary to the reporter signal such that replication produces tandem sequence DNA. The tandem sequence DNA contains a plurality of reporter tags.

The reporter molecules can also be amplified using oligonucleotide dendrimers. The decoding tags preferably are oligonucleotides, carbohydrates, peptide nucleic acids, antibodies, ligands, proteins, haptens, zinc fingers, aptamers, or mass labels.

In another embodiment, the target molecules can be homing molecules. Prior to step (a), the target samples or the source of the target samples are

exposed to the homing molecules. The result is that the presence of homing molecules corresponding to the detected decoding tags indicates the presence of molecules in the target samples to which the homing molecules are directed. For example, the source of the tissue samples can be exposed to the homing molecules such that the presence of homing molecules corresponding to the detected decoding tags indicates the presence of cells or molecules in the tissue samples to which the homing molecules are directed. For this embodiment, the target molecules preferably are tumor-homing peptides with the result that the presence of tumor-homing peptides corresponding to the detected decoding tags indicates the presence of tumor cells in the tissue samples. In preferred embodiments, the organisms that are the source of the tissue samples can be exposed to the homing molecules, or the tissue samples can be exposed to the homing molecules after the tissue samples are sectioned.

Where the target samples are cells, the cells can be sorted from other cells. For example, the cells are sorted based on the presence, absence, or difference in amount of a cell marker.

The disclosed method can be performed where the target samples are organisms, and where, following step (a), derivative target samples comprising the reporter molecules are prepared from the organisms. The disclosed method can also be performed where the target samples are tissues, and where, following step (a), derivative target samples comprising the reporter molecules are prepared from the tissues. The derivative target samples preferably are tissue sections prepared from the tissues.

In another embodiment, each decoding tag can correspond to a different reporter tag, each reporter tag can correspond to a different reporter molecule, each reporter molecule can correspond to a different target molecule, or a combination. Alternatively, each decoding tag can correspond to a single reporter tag, each reporter tag can correspond to a single reporter molecule, each reporter molecule can correspond to a single target molecule, or a combination. Or each decoding tag can correspond to multiple reporter tags, each reporter tag can correspond to multiple reporter molecules, each reporter molecule can correspond to multiple target molecules, or a combination.

The disclosed method can also include, following step (b), the step of bringing into contact the target samples and one or more capture arrays, where different target molecules become associated with different elements of the array such. The result is that the array elements with which the decoding tags are associated indicate the presence in the target samples of the target molecules corresponding to that array element. The capture array can comprise capture tags, where each array element comprises a different capture tag. The capture tags preferably are oligonucleotides, antibodies, haptens, ligands, or a combination. The capture array preferably comprises a substrate comprising beads, plates, or slides.

The decoding tags can be distinguished temporally via different fluorescent, phosphorescent, or chemiluminescent emission lifetimes. The decoding tags preferably are detectable by nuclear magnetic resonance, electron paramagnetic resonance, surface enhanced raman scattering, surface plasmon resonance, fluorescence, phosphorescence, chemiluminescence, resonance raman, microwave, mass spectrometry, or a combination. The decoding tags preferably are detected by mass spectrometry, electrophoresis, or chromatography.

In another embodiment, the decoding tags can be peptide nucleic acids, and the decoding tags can be detected by mass spectrometry. For this, the different decoding tags should differ in mass. In this embodiment, each reporter molecule can comprise a specific binding molecule, where the specific binding molecule of each reporter molecule interacts with a different target molecule, and the reporter tags can be oligonucleotides, where the decoding tags are peptide nucleic acids that are complementary to the reporter tags. Alternatively, each decoding tag can have the same number of nucleotide bases complementary to the reporter tag. In this case, it is preferred that each decoding tag comprises a different number of 8-amino-3,6-dioxaoctanoic monomers and that the decoding tags are detected by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy.

The decoding tags can also be fluorescently-labeled oligonucleotides, where each decoding tag has a different combination of length and fluorescent label. In this case, it is preferred that each reporter tag be an oligonucleotide,

and that each decoding tag have the same number of nucleotides complementary to the reporter tag. For this combination, it is preferred that each decoding tag that has the same fluorescent label has a different number of nucleotides not complementary to the reporter tag and that the decoding tags are detected by microdissection of the target sample and electrophoresis of the microdissected samples.

The target molecules can be in one or more target samples, where the target molecules include different modification states of the same target molecules. For example, the modifications can be fragmentation, cleavage, phosphorylation, glycosylation, methylation, alkylation, dimerization, derivatization, depurination, conformation, or ribosylation. Where the target molecules include different phosphorylation states of the same proteins, each of the reporter molecules can interact with a different protein in a different phosphorylation state, and detection of the target molecules in the target sample is indicative of the phosphorylation state of the proteins in the target sample. Where the target molecules include different glycosylation states of the same proteins, each of the reporter molecules can interact with a different protein in a different glycosylation state, and detection of the target molecules in the target sample is indicative of the glycosylation state of the proteins in the target sample. Where the target molecules include different poly-ADP ribosylation states of the same proteins, each of the reporter molecules can interact with a different protein in a different poly-ADP ribosylation state, and detection of the target molecules in the target sample is indicative of the poly-ADP ribosylation state of the proteins in the target sample. Where the target molecules include different fragments of the same proteins, each of the reporter molecules can interact with a different fragment, and detection of the target molecules in the target sample is indicative of the fragments of the proteins in the target sample. Where the target molecules include different conformational states of the same proteins, each of the reporter molecules can interact with a different protein in a different conformational state, and detection of the target molecules in the target sample is indicative of the conformational state of the proteins in the target sample. Where at least one of the target molecules is a prion protein, the

conformation states can include the prion conformation of a protein and the non-prion conformation of the protein.

The pattern of the presence, amount, presence and amount, or absence of decoding tags can constitute a catalog of the target molecules. Where the target molecules are in two or more target samples, the pattern of the presence, amount, presence and amount, or absence of decoding tags associated with each target sample constitutes a catalog of the target molecules in that target sample, the method further comprising comparing one or more catalogs with one or more other catalogs.

The target molecules, reporter molecules, or decoding tags can be in an array, where each target molecule, reporter molecule, or decoding tag is immobilized at a different location in the array, and where detecting the decoding tags is accomplished by detecting the presence, amount, presence and amount, or absence of decoding tags in the arrays. Where the location, amount, or location and amount of decoding tags in the arrays constitutes a pattern of decoding tags in the arrays, the pattern of decoding tags in the arrays can be compared with the pattern of decoding tags in arrays determined in a separate procedure using a different one or more target molecules. The pattern of decoding tags in the arrays can also be compared with the pattern of decoding tags in arrays determined in a plurality of separate procedures using a plurality of different one or more target molecules.

Where the target molecules are associated with cells, the reporter molecules can be associated with the target molecules. If each reporter molecule comprises a sorting tag, the cells can be sorted based on the sorting tags. The target molecules preferably are cell surface proteins on cells such that the reporter molecules are associated with the proteins on the cells.

Where each reporter molecule comprises a specific binding molecule, the specific binding molecule preferably is an antibody, a ligand, a binding protein, a receptor protein, a hapten, aptamer, carbohydrate, or an oligonucleotide. Where the specific binding molecules is a binding protein, it preferably is a DNA binding protein comprising one or more zinc finger motifs, leucine zipper motifs, helix-turn-helix motifs, or a combination.

The decoding tags preferably are detected by determining the presence, amount, presence and amount, or absence of the decoding tags. The decoding tags can be separated and detected by high pressure liquid chromatography. The target sample can be immobilized, fixed, or adhered to a surface such that the locations of the target molecules that are determined are the locations of the target molecules on the surface.

Correspondence of Components

In preferred embodiments, a one-to-one-to-one correspondence between target molecules, reporter molecules, and decoding tags is used. In this way, each different type of target molecule ends up with a different and separately detectable decoding tag associated with it. The combination of specific binding molecule and reporter signal results in an effective conversion of the different target molecules (where the target molecules may be widely chemically divergent) into a standardized signal (the reporter signal). These standardized signals (which are coded for each target molecule) are then detected using a single set of standardized conditions (during which the coding is carried forward). The detection of the coded signals (that is, the decoding tags) results in effective, convenient detection of multiple target molecules in a single assay.

Target molecules, reporter molecules, reporter tags, and decoding tags can be mapped to each other in a variety of different ways. For example, each decoding tag can correspond to a different reporter tag, each reporter tag can correspond to a different reporter molecule, each reporter molecule can correspond to a different target molecule, or there can be a combination of these relationships. Each decoding tag can correspond to a single reporter tag, each reporter tag can correspond to a single reporter molecule, each reporter molecule can correspond to a single target molecule, or there can be a combination of these relationships. Each decoding tag can correspond to multiple reporter tags, each reporter tag can correspond to multiple reporter molecules, each reporter molecule can correspond to multiple target molecules, or there can be a combination of these relationships. Many other combinations of relationships are also possible.

Decoding Tag Detection

Decoding tags can be detected using any suitable technique. In general, the properties of the decoding tags will be chosen to match, or be compatible with, a chosen detection technique. In preferred embodiments, the disclosed method uses rapid detection techniques that allow spatial information about analytes to be gathered. Decoding tags preferably are detected by nuclear magnetic resonance, electron paramagnetic resonance, surface enhanced raman scattering, surface plasmon resonance, fluorescence, phosphorescence, chemiluminescence, resonance raman, microwave, mass spectrometry, or any combination of these. Decoding tags preferably are separated and/or detected by mass spectrometry, electrophoresis, or chromatography. Decoding tags can be distinguished temporally via different fluorescent, phosphorescent, or chemiluminescent emission lifetimes.

Preferred methods of detection allow the location and amount of the decoding tags to be determined. Preferred techniques to accomplish this include detection by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy and detection by microdissection of the target sample and capillary electrophoresis of the microdissected samples.

For MALDI-TOF detection, the decoding tags preferably are peptide nucleic acids, where each decoding tag has a different mass to allow separation and separate detection in mass spectroscopy. For this purpose, it is preferable that each decoding tag have the same number of nucleotide bases complementary to the reporter tag. It is also preferable that each decoding tag have a different number of mass tags, such as 8-amino-3,6-dioxaoctanoic monomers. This allows for more consistent hybridization characteristics while allowing the mass to vary. Use of peptide nucleic acids in MALDI-TOF detection is generally described by Baucom et al., Anal. Chem. 69:4894-4898 (1997), and Butler et al., Anal. Chem. 68:3283-3287 (1996).

For capillary electrophoresis detection, the decoding tags preferably are fluorescently-labeled oligonucleotides, where each decoding tag has a different combination of length and fluorescent label. For this purpose, it is preferable that each decoding tag have the same number of nucleotides complementary to the reporter tag. It is also preferable that each decoding tag have a different

number of nucleotides not complementary to the reporter tag. This allows for more consistent hybridization characteristics while allowing separation of the different decoding tags during electrophoresis.

The disclosed method using MALDI-TOF detection can be summarized as follows:

1. A mixture of different reporter molecules is contacted with a sample to allow specific binding of reporter molecules to target molecules on the surface of the sample; then excess unbound reporter molecules are removed.

2. A nucleic acid amplification system is used to generate surface-bound reporter tags, such that reporter tag abundance is proportional to the number of bound reporter molecules.

3. The surface is contacted with a mixture of decoding tags. The decoding tags can be distinguished by molecular weight because they are of different composition and/or contain different mass labels. The mixture of decoding tags is allowed to bind to the reporter tags; then the excess of decoding tags is removed.

4. The surface is processed for mass spectrometry by coating it with a suitable matrix for laser-desorption ionization.

5. The slide is introduced in a mass spectrometer, and the laser is directed successively to specific locations of the surface in order to obtain desorption-ionization in specific areas, releasing the decoding tags. The amount of each decoding tag of different mass is measured, providing a relative measure of the number of bound reporter molecules of each specific class. The laser desorption process is then repeated on many different locations on the surface.

Amplification of Reporter Molecules

Reporter molecules can be amplified using any suitable technique. Many amplification techniques are known and can be adapted for use in the disclosed method. The form of amplification will generally be related to the nature of the reporter molecule, and in particular, to the nature of the reporter signal to be amplified. A major class of amplification techniques are nucleic acid amplification techniques. Such techniques generally will be most useful when the reporter signal is an oligonucleotide or other nucleic acid or nucleic acid derivative. As used herein, amplification of a reporter molecule refers to any

increase in signal (in the form of reporter tags) associated with a reporter molecule over the signal (in the form of a reporter signal) that is part of a reporter molecule.

Reporter signals preferably are amplified using branched DNA, rolling circle amplification, or oligonucleotide dendrimers. The branched DNA generally has a tail complementary to the reporter signal and also has multiple reporter tags. Use of branched DNA for signal amplification is generally described by Urdea, *Biotechnology* 12:926-928 (1994), and Horn *et al.*, *Nucleic Acids Res* 23:4835-4841 (1997). Use of dendrimers for signal amplification is generally described by Shchepinov et al., Nucleic Acids Res. 25:4447-4454 (1997), and Orentas et al., J. Virol. Methods 77:153-163 (1999). For rolling circle amplification, the reporter signal primes rolling circle replication of an amplification target circle. Since an amplification target circle comprises one or more sequences complementary to the reporter tag, replication produces tandem sequence DNA where the tandem sequence DNA contains a plurality of reporter tags. Rolling circle replication is described in U.S. Patent No. 5,854,033.

Analysis of Modification States

Molecules can exist in a variety of states. For example, proteins can have different phosphorylation states. The disclosed method can be used to analyze the state of various modifications of target molecules in target samples. Such modifications include fragmentation, cleavage, phosphorylation, glycosylation, methylation, alkylation, dimerization, derivatization, depurination, conformation, or ribosylation. In basic form, this can be accomplished by interacting different target molecules in different modification states with different reporter molecules specific for the different modification states.

An example of this is the generation of multiplexed profiles of phosphorylated proteins. In this embodiment, the status of phosphorylated versus non-phosphorylated forms of proteins (or between different phosphorylation states of proteins) can be monitored through the use of a multiplicity of sets of antibodies, with each specific for a different phosphorylation state of a protein. The decoding tag signals obtained by electrophoretic analysis or by MALDI-TOF spectrometry are interpreted to yield

ratios of the various phosphorylation states for a multiplicity of proteins, and this information can then be correlated with the physiological status of the cell. This embodiment preferably is used with proteins involved in signal transduction. Examples of proteins and antibodies suitable for this embodiment are described by Gioeli et al., Cancer Res 59(2):279-84 (1999), and Ng et al., Science 283(5410):2085-2089 (1999). In a similar way, the disclosed method can be used to multiplex profiles of the fragmentation, cleavage, glycosylation, methylation, alkylation, dimerization, derivatization, depurination, conformation, or ribosylation of target molecules.

A number of peptide cleavage means are known (Means, G.E. and R.E. Feeney, *Chemical modification of proteins*. 1971, San Francisco,: Holden-Day. x, 254; Lundblad, R.L., *Chemical reagents for protein modification*. 2nd ed. 1991, Boca Raton: CRC Press. 345.) and may be utilized in solution, on surfaces, or in a matrix. Some examples include: cleavage of methionine-containing peptide bonds with cyanogen bromide for specific cleavage at the carboxyl side, cleavage of cysteine containing peptide bonds by conversion of cysteine to dehydroalanine and subsequent hydrolysis, cleavage of tryptophan-containing peptide bonds at the amino side with N-chlorosuccinimide, and cleavage of asparagine-glycine-containing peptide bonds by hydroxylamine with specific cleavage at the carboxyl side.

Changes in protein conformation recognized by antibodies can be assessed using such antibodies. Prions are proteins capable of self-perpetuating changes in conformation and function. They have been implicated in several biological processes and diseases such as Alzheimers (Prusiner, S.B., *Prions.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13363-83; Li, L. and S. Lindquist, *Creating a protein-based element of inheritance.* Science, 2000. **287**(5453): p. 661-4). Antibodies have been developed that recognize the different states of prion proteins (Hardt, M., T. Baron, and M.H. Groschup, *A comparative study of immunohistochemical methods for detecting abnormal prion protein with monoclonal and polyclonal antibodies.* J Comp Pathol, 2000. **122**(1): p. 43-53; Korth, C., P. Streit, and B. Oesch, *Monoclonal antibodies specific for the native, disease-associated isoform of the prion protein.* Methods Enzymol, 1999. **309**: p. 106-22).

Sample Processing

Target samples can be processed in a variety of ways both before and after association of reporter molecules. In the usual case, a target sample will be separated or purified from a source of interest. For example, cells can be isolated from an organism or tissue, tissue can be isolated from an organism, or cellular contents or components can be isolated from cells. Other processing is possible. For example, cells, tissue, or organisms can be exposed to reporter molecules prior to isolation of preparation of a target sample. Where the target samples are processed before continuing or completing the method, the processed target sample can be referred to as a derivative target sample. Unless otherwise indicated, the term target sample refers to both target samples and derivative target samples.

A target sample, or the source of a target sample, also can be "stained" with target molecules prior to exposure to reporter molecules or prior to sample preparation. In this embodiment, the target molecules become associated with some other molecules or features of the sample. For example, a tissue sample can be stained with target molecules that bind to particular proteins of interest. Such target molecules are referred to herein as homing molecules. The presence and location to these proteins can then be determined in the disclosed method by associating reporter molecules with the target molecules. A similar effect can be achieved by staining a target sample with reporter molecules themselves.

A tissue sample can be exposed to homing molecules either before or after the tissue samples are sectioned. In a preferred embodiment the target molecules are tumor-homing peptides. The presence of tumor-homing peptides corresponding to the detected decoding tags indicates the presence of tumor cells in the tissue samples.

Where the target samples are cells, or are derived from cells, the cells can be sorted from other cells. In particular, the cells can be sorted based on the presence, absence, or difference in amount of a cell marker. FACS is a useful technique for accomplishing such cell sorting. In some embodiments, a sorting tag can be included on the reporter molecules and sorting can be accomplished using the sorting tags.

In some embodiments of the method, cells may be sorted prior to preparation of the target sample or association with reporter molecules. For example, cells in blood, such as peripheral lymphocytes and macrophages, may be sorted into different sub-populations using a fluorescence-activated cell sorter. Sorting may be based on the presence or absence, or on measurably different amounts of certain cell surface markers such as proteins or carbohydrates. After cell sorting, the cell sub-populations may be deposited in individual microtiter wells, or may be placed on the surface of a suitably prepared microscope slide, where they may be fixed using conventional cytology procedures. Sorted cells are ideally suited for analysis in the disclosed method since they represent a relatively homogeneous sub-population, where the relative ratios of cellular components such as proteins or mRNA's are likely to be quite similar in every cell.

Cell sorting can also be done during the disclosed method. Thus, cells of the immune system may be sorted according to cell surface markers such as CD subtype. An analysis may be set up where a subset of the reporter molecules includes an additional signal moiety whose purpose is to enable a cell sorting step. A preferred additional signal moiety for this embodiment is a fluorescent dye. For example, the dye cy3 may be covalently associated with a specific DNA reporter tag, the tag being covalently bound to an antibody specific for the cell surface determinant CD4. Likewise, the dye cy5 may be covalently associated with a different specific DNA reporter tag, the tag being covalently bound to an antibody specific for the cell surface determinant CD8. A set of 18 other specific antibodies (directed against other cellular proteins, which may be either internal or surface proteins) is covalently associated with 18 different DNA reporter tags. These tags, however, do not contain fluorescent dyes. The set of 20 reporter molecules, which comprises 18 non-fluorescent reporters and 2 fluorescent reporters, is mixed with a population of cells from human blood. The cells are then sorted on the basis of cy3 and cy5 fluorescence, using a fluorescence-activated cell sorter, to obtain three different cell pools: High CD4, High CD8, and everything else. These pools of cells are then separately subjected to multiple tag analysis for all 20 antibody reporter systems. In this analysis step, the CD4 and CD8 signals, as well as the 18 other signals, are

obtained simultaneously based on the amount of specific decoding tags detected by mass spectrometry. Such analysis will generate a catalog of protein expression levels for each of the three cell pools.

Target samples and target molecules can also be immobilized on substrates. Preferably, target molecules are associated with capture arrays where different target molecules become associated with different elements of the array. In this way, the identity of the array element where decoding tags are detected identifies the target molecule involved. Generally, association of target molecules to a capture array will be through association with capture tags immobilized on the substrate. Preferred forms of capture tags are oligonucleotides, antibodies, haptens, and ligands.

The disclosed method may be used for the detection of a panel of defined analytes that together are indicative of the metabolic status of tissue. For example, one may obtain a tissue biopsy form an experimental animal to assay for the possible presence of cancer. Tissue sections are prepared, and analyzed with a plurality of oligonucleotide-tagged antibodies, said tagged antibodies being specific for binding to specific tumor antigens, chemokines, cytokines, and tumor-homing peptides (Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis, *Cancer Res* 2000 60:722-727; Pasqualini R. Vascular targeting with phage peptide libraries, *Q J Nucl Med* 1999 43:159-162). A tumor-homing peptide is an oligopeptide that has been selected on the basis of its binding specificity for a unique type of tumor. By injecting the experimental animal with an equimolar mixture of a plurality of tumor-homing peptides, the various tissues of the animal will retain the peptides depending on the presence or absence of tumor cells. The actual concentration of tumor-homing peptides bound in tumor tissue will vary according to the stage and the grade of the tumor. By using a mixture of tumor-homing peptides, and employing the histological analysis of the method, a profile of the relative ratios of an entire panel of tumor-homing peptides at any number of locations in a tissue section can be obtained. These multiple analyte profiles derived from the retention of injected peptides in tissue, in combination with the relative levels of endogenous

tumor markers, growth factors, cytokines, etc., may be correlated with the disease status of the animal.

Patterns and Catalogs of Target Molecules

Patterns of the presence, amount, presence and amount, or absence of decoding tags constitute a catalog of the target molecules. Such catalogs can be used as fingerprints of target samples, and are particularly useful for comparison with other catalogs derived from different targets samples in the same manner. Where the target molecules, reporter molecules, or decoding tags are in an array, the presence, amount, presence and amount, or absence of decoding tags in the array constitute a pattern of decoding tags in the array. Such a pattern can be compared to the pattern of decoding tags in arrays determined in a separate procedure using a different one or more target molecules. Multiple patterns derived from multiple target samples can be compared in this way.

Illustration 1: Protein Detection Using Branched DNA Amplification and MALDI-TOF Detection

The following illustrates an embodiment of the disclosed method used to detect proteins where the reporter signals are amplified using branched DNA and where the decoding tags are detected using MALDI-TOF.

1. A branch-DNA assay is performed on the surface of a glass slide containing a standard histological specimen of paraformaldehyde-fixed tissue or a cytological specimen, with the objective of detecting four different proteins on the surface of the histological or cytological sample. Four different antibodies (the specific binding molecules; A, B, C, D) are used, each specific for a specific protein of interest. Each antibody is covalently coupled to a specific oligonucleotide (the reporter signal; Ao1, Bo2, Co3, Do4), providing each antibody with a specific DNA tag that serves as a binding site for the branch-DNA. Four different branch-DNA reporters are used (Ao1-Br1, Bo2-Br2, Co3-Br3, Do4-Br4) each reporter containing a tree of at least 1,000 DNA reporter tags. After completion of the branch-DNA assay, the surface will contain thousands of bound antibody molecules, and each antibody molecule will be bound (via the reporter signal) by its own specific tree of branch-DNA. If the distribution of the four proteins in tissue is non-homogenous, then the

distribution of surface-bound antibodies will, of course, be non-homogeneous. Consequently, the surface distribution of the thousands of strands of branch-DNA reporter tags Br1, Br2, Br3, and Br4 will also be non-homogeneous.

2. The surface is contacted with a mixture of four different peptide nucleic acid (PNA) decoding tags (PNA1, PNA2, PNA3, PNA4). The PNAs are of the same length (10 bases), but comprise four different sequences perfectly complementary to each of the reporter tag sequences. Additionally, the PNAs can be distinguished by molecular weight because they contain one, two, three, or four NH_2-terminal 8-amino-3,6-dioxaoctanoic monomers (146 Daltons each) as a mass labels for MALDI-TOF. The mixture of four PNAs is allowed to hybridize for 15 minutes on the surface of the glass slide, and then the slide is washed to remove unhybridized PNA.

3. The slide is covered with a thin layer of a 10:1:1 mixture of 3-hydroxypicolinic acid, picolinic acid, and diammonium citrate (Smirnov et al, Analytical Biochemistry 238:19 (1996)) and then is allowed to dry in order to embed the reporter tags, together with PNA decoding tags, within a matrix of small molecules suitable for optimal time-delayed desorption.

4. The slide is introduced in a mass spectrometer, and the laser is directed to specific locations on the tissue order to obtain laser desorption in specific areas- of approximately 500 square microns, releasing the PNA molecules (Figure 1.3). The MALDI-TOF mass spectrum is interpreted to document the amount of each of the four PNA decoding tags (PNA1, PNA2, PNA3, PNA4), thus measuring indirectly the relative amounts of each protein (A, B, C, D). Individual measurements may be performed sequentially at different surface locations, since the laser desorption time for each spot is rapid (on the order of seconds).

Illustration 2: Protein Detection Using Rolling Circle Amplification and MALDI-TOF Detection

The following illustrates an embodiment of the disclosed method used to detect proteins where the reporter signals are amplified using rolling circle amplification and where the decoding tags are detected using MALDI-TOF.

1. Rolling circle amplification is performed on the surface of a glass slide containing a standard histological specimen of paraformaldehyde-fixed

tissue or a cytological specimen, with the objective of detecting four different proteins on the surface of the histological or cytological sample. Four different antibodies (the specific binding molecules; A, B, C, D) are used, each specific for a specific protein of interest. Each antibody is covalently coupled to a specific oligonucleotide (the reporter signal; Ao1, Bo2, Co3, Do4), providing each antibody with a specific DNA tag that serves as a primer for rolling circle amplification. Four different amplification target circles are used (Ao1-ATC1, Bo2-ATC2, Co3-ATC3, Do4-ATC4) each amplification target circle containing one or more reporter tag complements. The amplification target circles are replicated using the reporter signals as the rolling circle replication primer to form tandem sequence DNA containing at least 1,000 DNA reporter tags. After completion of rolling circle amplification, the surface will contain thousands of bound antibody molecules, and each antibody molecule will be coupled (via the reporter signal) to its own specific tandem sequence DNA (TS1, TS2, TS3, TS4). If the distribution of the four proteins in tissue is non-homogenous, then the distribution of surface-bound antibodies will, of course, be non-homogeneous. Consequently, the surface distribution of the tandem sequence DNA reporter tags TS1, TS2, TS3, and TS4 will also be non-homogeneous.

2. The surface is contacted with a mixture of four different peptide nucleic acid (PNA) decoding tags (PNA1, PNA2, PNA3, PNA4). The PNAs are of the same length (10 bases), but comprise four different sequences perfectly complementary to each of the reporter tag sequences. Additionally, the PNAs can be distinguished by molecular weight because they contain one, two, three, or four NH_2-terminal 8-amino-3,6-dioxaoctanoic monomers (146 Daltons each) as a mass labels for MALDI-TOF. The mixture of four PNAs is allowed to hybridize for 15 minutes on the surface of the glass slide, and then the slide is washed to remove unhybridized PNA.

3. The slide is covered with a thin layer of a 10:1:1 mixture of 3-hydroxypicolinic acid, picolinic acid, and diammonium citrate (Smirnov et al, Analytical Biochemistry 238:19 (1996)) and then is allowed to dry in order to embed the reporter tags, together with PNA decoding tags, within a matrix of small molecules suitable for optimal time-delayed desorption.

4. The slide is introduced in a mass spectrometer, and the laser is directed to specific locations on the tissue order to obtain laser desorption in specific areas- of approximately 500 square microns, releasing the PNA molecules (Figure 1.3). The MALDI-TOF mass spectrum is interpreted to document the amount of each of the four PNA decoding tags (PNA1, PNA2, PNA3, PNA4), thus measuring indirectly the relative amounts of each protein (A, B, C, D). Individual measurements may be performed sequentially at different surface locations, since the laser desorption time for each spot is rapid (on the order of seconds).

Illustration 3: Messenger RNA Detection Using Branched DNA Amplification and Capillary Electrophoresis Detection

The following illustrates an embodiment of the disclosed method used to detect mRNA where the reporter signals are amplified using branched DNA and where the decoding tags are detected using capillary electrophoresis.

1. A branch-DNA assay is performed on the surface of a glass slide containing a standard histological specimen of paraformaldehyde-fixed tissue or a cytological specimen, with the objective of detecting four different mRNA on the surface of the histological or cytological sample. Four different mRNA probes (the specific binding molecules; A, B, C, D) are used, each specific for a specific mRNA of interest. Each probe is covalently coupled to a specific oligonucleotide (the reporter signal; Ao1, Bo2, Co3, Do4), providing each mRNA probe with a specific DNA tag that serves as a binding site for the branch-DNA. Four different branch-DNA reporters are used (Ao1-Br1, Bo2-Br2, Co3-Br3, Do4-Br4) each reporter containing a tree of at least 1,000 DNA reporter tags. After completion of the branch-DNA assay, the surface will contain thousands of bound probe molecules, and each probe molecule will be bound (via the reporter signal) by its own specific tree of branch-DNA. If the distribution of the four mRNAs in tissue is non-homogenous, then the distribution of surface-bound probes will, of course, be non-homogeneous. Consequently, the surface distribution of the thousands of strands of branch-DNA reporter tags Br1, Br2, Br3, and Br4 will also be non-homogeneous (Figure 1.1).

2. The surface is contacted with a mixture of four different fluorescently-labeled DNA tags (decoding tags; DNA1, DNA2, DNA3, DNA4), which are complementary to each of the four different reporter tag sequences. The decoding tags are of different lengths (20, 21, 22, and 23 bases) where only 20 bases in each are complementary to the reporter tags. The additional bases serve to differentiate the mass of the decoding tags. The fluorescent DNA tags are allowed to hybridize for 20 minutes on the surface of the glass slide, and then the slide is washed to remove unhybridized decoding tags (Figure 1.2, heavier lines indicate larger DNAs).

3. The slide is place on an Arcturus Engineering laser dissection microscope, and different areas of interest containing anywhere from 1 cell to 1,000 cells are microdissected.

4. The microdissected cellular material, bound on the plastic cap of the laser microdissection microscope, is dissolved in 40 μl of 90% formamide gel loading buffer. The material is transferred to another tube, and then loaded into a capillary electrophoresis instrument by contacting the tip of the capillary with the small 40 μl sample, and turning on the voltage.

5. The profile of fluorescent decoding tags is recorded, and the different peak intensities are correlated with the corresponding tag in order to generate the relative expression profiles for the mRNA analytes that were recognized by each probe.

Illustration 4: Messenger RNA Detection Using Rolling Circle Amplification and Capillary Electrophoresis Detection

The following illustrates an embodiment of the disclosed method used to detect mRNA where the reporter signals are amplified using rolling circle amplification and where the decoding tags are detected using capillary electrophoresis.

1. Rolling circle amplification is performed on the surface of a glass slide containing a standard histological specimen of paraformaldehyde-fixed tissue or a cytological specimen, with the objective of detecting four different mRNA on the surface of the histological or cytological sample. Four different mRNA probes (the specific binding molecules; A, B, C, D) are used, each specific for a specific mRNA of interest. Each probe is covalently coupled to a

specific oligonucleotide (the reporter signal; Ao1, Bo2, Co3, Do4), providing each mRNA probe with a specific DNA tag that serves as a primer for rolling circle amplification. Four different amplification target circles are used (Ao1-ATC1, Bo2-ATC2, Co3-ATC3, Do4-ATC4) each amplification target circle containing one or more reporter tag complements. The amplification target circles are replicated using the reporter signals as the rolling circle replication primer to form tandem sequence DNA containing at least 1,000 DNA reporter tags. After completion of rolling circle amplification, the surface will contain thousands of bound mRNA probes, and each mRNA probe will be coupled (via the reporter signal) to its own specific tandem sequence DNA (TS1, TS2, TS3, TS4). If the distribution of the four mRNAs in tissue is non-homogenous, then the distribution of surface-bound mRNA probes will, of course, be non-homogeneous. Consequently, the surface distribution of the tandem sequence DNA reporter tags TS1, TS2, TS3, and TS4 will also be non-homogeneous.

2. The surface is contacted with a mixture of four different fluorescently-labeled DNA tags (decoding tags; DNA1, DNA2, DNA3, DNA4), which are complementary to each of the four different reporter tag sequences. The decoding tags are of different lengths (20, 21, 22, and 23 bases) where only 20 bases in each are complementary to the reporter tags. The additional bases serve to differentiate the mass of the decoding tags. The fluorescent DNA tags are allowed to hybridize for 20 minutes on the surface of the glass slide, and then the slide is washed to remove unhybridized decoding tags (Figure 1.2, heavier lines indicate larger DNAs)

3. The slide is place on an Arcturus Engineering laser dissection microscope, and different areas of interest containing anywhere from 1 cell to 1,000 cells are microdissected.

4. The microdissected cellular material, bound on the plastic cap of the laser microdissection microscope, is dissolved in 40 µl of 90% formamide gel loading buffer. The material is transferred to another tube, and then loaded into a capillary electrophoresis instrument by contacting the tip of the capillary with the small 40 µl sample, and turning on the voltage.

5. The profile of fluorescent decoding tags is recorded, and the different peak intensities are correlated with the corresponding tag in order to generate the

relative expression profiles for the mRNA analytes that were recognized by each probe.

Illustration 5: Protein Detection Using Branched DNA Amplification and Capillary Electrophoresis Detection

The following illustrates an embodiment of the disclosed method used to detect proteins where the reporter signals are amplified using branched DNA and where the decoding tags are detected using capillary electrophoresis.

1. A branch-DNA assay is performed on the surface of a glass slide containing a standard histological specimen of paraformaldehyde-fixed tissue or a cytological specimen, with the objective of detecting four different proteins on the surface of the histological or cytological sample. Four different antibodies (the specific binding molecules; A, B, C, D) are used, each specific for a specific protein of interest. Each antibody is covalently coupled to a specific oligonucleotide (the reporter signal; Ao1, Bo2, Co3, Do4), providing each antibody with a specific DNA tag that serves as a binding site for the branch-DNA. Four different branch-DNA reporters are used (Ao1-Br1, Bo2-Br2, Co3-Br3, Do4-Br4) each reporter containing a tree of at least 1,000 DNA reporter tags. After completion of the branch-DNA assay, the surface will contain thousands of bound antibody molecules, and each antibody molecule will be bound (via the reporter signal) by its own specific tree of branch-DNA. If the distribution of the four proteins in tissue is non-homogenous, then the distribution of surface-bound antibodies will, of course, be non-homogeneous. Consequently, the surface distribution of the thousands of strands of branch-DNA reporter tags Br1, Br2, Br3, and Br4 will also be non-homogeneous.

2. The surface is contacted with a mixture of four different fluorescently-labeled DNA tags (decoding tags; DNA1, DNA2, DNA3, DNA4), which are complementary to each of the four different reporter tag sequences. The decoding tags are of different lengths (20, 21, 22, and 23 bases) where only 20 bases in each are complementary to the reporter tags. The additional bases serve to differentiate the mass of the decoding tags. The fluorescent DNA tags are allowed to hybridize for 20 minutes on the surface of the glass slide, and then the slide is washed to remove unhybridized decoding tags (Figure 1.2, heavier lines indicate larger DNAs)

3. The slide is place on an Arcturus Engineering laser dissection microscope, and different areas of interest containing anywhere from 1 cell to 1,000 cells are microdissected.

4. The microdissected cellular material, bound on the plastic cap of the laser microdissection microscope, is dissolved in 40 µl of 90% formamide gel loading buffer. The material is transferred to another tube, and then loaded into a capillary electrophoresis instrument by contacting the tip of the capillary with the small 40 µl sample, and turning on the voltage.

5. The profile of fluorescent decoding tags is recorded, and the different peak intensities are correlated with the corresponding tag in order to generate the relative expression profiles for the protein analytes that were recognized by each antibody.

Illustration 6: Protein Detection Using Rolling Circle Amplification and Capillary Electrophoresis Detection

The following illustrates an embodiment of the disclosed method used to detect proteins where the reporter signals are amplified using rolling circle amplification and where the decoding tags are detected using capillary electrophoresis.

1. Rolling circle amplification is performed on the surface of a glass slide containing a standard histological specimen of paraformaldehyde-fixed tissue or a cytological specimen, with the objective of detecting four different proteins on the surface of the histological or cytological sample. Four different antibodies (the specific binding molecules; A, B, C, D) are used, each specific for a specific protein of interest. Each antibody is covalently coupled to a specific oligonucleotide (the reporter signal; Ao1, Bo2, Co3, Do4), providing each antibody with a specific DNA tag that serves as a primer for rolling circle amplification. Four different amplification target circles are used (Ao1-ATC1, Bo2-ATC2, Co3-ATC3, Do4-ATC4) each amplification target circle containing one or more reporter tag complements. The amplification target circles are replicated using the reporter signals as the rolling circle replication primer to form tandem sequence DNA containing at least 1,000 DNA reporter tags. After completion of rolling circle amplification, the surface will contain thousands of bound antibody molecules, and each antibody molecule will be coupled (via the

reporter signal) to its own specific tandem sequence DNA (TS1, TS2, TS3, TS4). If the distribution of the four proteins in tissue is non-homogenous, then the distribution of surface-bound antibodies will, of course, be non-homogeneous. Consequently, the surface distribution of the tandem sequence DNA reporter tags TS1, TS2, TS3, and TS4 will also be non-homogeneous.

2. The surface is contacted with a mixture of four different fluorescently-labeled DNA tags (decoding tags; DNA1, DNA2, DNA3, DNA4), which are complementary to each of the four different reporter tag sequences. The decoding tags are of different lengths (20, 21, 22, and 23 bases) where only 20 bases in each are complementary to the reporter tags. The additional bases serve to differentiate the mass of the decoding tags. The fluorescent DNA tags are allowed to hybridize for 20 minutes on the surface of the glass slide, and then the slide is washed to remove unhybridized decoding tags (Figure 1.2, heavier lines indicate larger DNAs)

3. The slide is place on an Arcturus Engineering laser dissection microscope, and different areas of interest containing anywhere from 1 cell to 1,000 cells are microdissected.

4. The microdissected cellular material, bound on the plastic cap of the laser microdissection microscope, is dissolved in 40 μl of 90% formamide gel loading buffer. The material is transferred to another tube, and then loaded into a capillary electrophoresis instrument by contacting the tip of the capillary with the small 40 μl sample, and turning on the voltage.

5. The profile of fluorescent decoding tags is recorded, and the different peak intensities are correlated with the corresponding tag in order to generate the relative expression profiles for the protein analytes that were recognized by each antibody.

CLAIMS

We claim:

1. A method of detecting multiple target molecules in a sample, the method comprising

(a) bringing into contact one or more target samples and one or more reporter molecules,

wherein the reporter molecules are associated with target molecules in the target samples,

(b) amplifying the reporter molecules to produce multiple reporter tags for each reporter molecule, wherein the reporter tags remain associated with the reporter molecule,

(c) associating one or more decoding tags with the reporter tags,

(d) detecting the decoding tags, *wherein the decoding tags are disassociated from the reporter molecules during, or prior to, detection,* wherein the decoding tags correspond to reporter molecules, wherein the reporter molecules correspond to target molecules, wherein detecting the decoding tags indicates the presence of reporter molecules corresponding to the detected decoding tags, wherein the presence of reporter molecules indicates the presence of target molecules corresponding to the reporter molecules.

2. The method of claim 1 wherein each reporter molecule comprises one or more specific binding molecules, wherein the specific binding molecule of each reporter molecule interacts with a different target molecule, wherein the reporter tags produced from each reporter molecule are different from the reporter tags produced from other reporter molecules, wherein a different decoding tag corresponds to each different reporter tag.

3. The method of claim 1 wherein the reporter molecules are amplified using branched DNA.

4. The method of claim 3 wherein each reporter molecule comprises a reporter signal, wherein each branched DNA comprises multiple reporter tags and a tail which associates with the reporter signal.

5. The method of claim 1 wherein the reporter molecules are amplified using rolling circle amplification.

6. The method of claim 5 wherein each reporter molecule comprises a reporter signal, wherein the reporter signal primes rolling circle replication of an amplification target circle, wherein the amplification target circle comprises one or more sequences complementary to the reporter signal, wherein replication produces tandem sequence DNA, wherein the tandem sequence DNA contains a plurality of reporter tags.

7. The method of claim 1 wherein the reporter molecules are amplified using oligonucleotide dendrimers.

8. The method of claim 1 wherein the decoding tags are oligonucleotides, carbohydrates, peptide nucleic acids, synthetic polyamides, antibodies, ligands, proteins, haptens, zinc fingers, aptamers, or mass labels.

9. The method of claim 1 wherein the target molecules are homing molecules, wherein, prior to step (a), the target samples or the source of the target samples are exposed to the homing molecules, wherein the presence of homing molecules corresponding to the detected decoding tags indicates the presence of molecules in the target samples to which the homing molecules are directed.

10. The method of claim 9 wherein the target samples are tissue samples, wherein, prior to step (a), the source of the tissue samples is exposed to the homing molecules, wherein the presence of homing molecules corresponding to the detected decoding tags indicates the presence of cells or molecules in the tissue samples to which the homing molecules are directed.

11. The method of claim 10 wherein the target molecules are tumor-homing peptides, wherein the presence of tumor-homing peptides corresponding to the detected decoding tags indicates the presence of tumor cells in the tissue samples.

12. The method of claim 10 wherein the organisms that are the source of the tissue samples are exposed to the homing molecules.

13. The method of claim 9 wherein the target samples are tissue samples, wherein the tissue samples are exposed to the homing molecules after the tissue samples are sectioned.

14. The method of claim 1 wherein the target samples are cells, wherein the cells have been sorted from other cells.

15. The method of claim 14 wherein the cells are sorted based on the presence, absence, or difference in amount of a cell marker.

16. The method of claim 1 wherein the target samples are organisms, wherein, following step (a), derivative target samples comprising the reporter molecules are prepared from the organisms.

17. The method of claim 1 wherein the target samples are tissues, wherein, following step (a), derivative target samples comprising the reporter molecules are prepared from the tissues.

18. The method of claim 17 wherein the derivative target samples are tissue sections prepared from the tissues.

19. The method of claim 1 wherein each decoding tag corresponds to a different reporter tag, each reporter tag corresponds to a different reporter molecule, each reporter molecule corresponds to a different target molecule, or a combination.

20. The method of claim 19 wherein each decoding tag corresponds to a single reporter tag, each reporter tag corresponds to a single reporter molecule, each reporter molecule corresponds to a single target molecule, or a combination.

21. The method of claim 19 wherein each decoding tag corresponds to multiple reporter tags, each reporter tag corresponds to multiple reporter molecules, each reporter molecule corresponds to multiple target molecules, or a combination.

22. The method of claim 1 further comprising, following step (b),

bringing into contact the target samples and one or more capture arrays, wherein different target molecules become associated with different elements of the array, wherein the array elements with which the decoding tags are associated indicate the presence in the target samples of the target molecules corresponding to that array element.

23. The method of claim 22 wherein the capture array comprises capture tags, wherein each array element comprises a different capture tag.

24. The method of claim 22 wherein the capture tags are oligonucleotides, antibodies, haptens, ligands, or a combination.

25. The method of claim 22 wherein the capture array comprises a substrate.

26. The method of claim 25 wherein the substrate comprises beads, plates, or slides.

27. The method of claim 1 wherein at least two of the decoding tags are distinguished temporally via different fluorescent, phosphorescent, or chemiluminescent emission lifetimes.

28. The method of claim 1 wherein the decoding tags are detectable by nuclear magnetic resonance, electron paramagnetic resonance, surface enhanced raman scattering, surface plasmon resonance, fluorescence, phosphorescence, chemiluminescence, resonance raman, microwave, mass spectrometry, or a combination.

29. The method of claim 1 wherein the decoding tags are detected by mass spectrometry, electrophoresis, or chromatography.

30. The method of claim 1 wherein the decoding tags are peptide nucleic acids, wherein the decoding tags are detected by mass spectrometry, wherein the different decoding tags differ in mass.

31. The method of claim 30 wherein each reporter molecule comprises a specific binding molecule, wherein the specific binding molecule of each reporter molecule interacts with a different target molecule, wherein the reporter tags are oligonucleotides, wherein the decoding tags are peptide nucleic acids that are complementary to the reporter tags.

32. The method of claim 31 wherein the specific binding molecules are oligonucleotides, wherein the specific binding molecules hybridize to the target molecules, wherein each of the specific binding molecules form a stem and loop when not hybridized to a target molecule, wherein the stem is disrupted when the specific binding molecules hybridize to the target molecules.

33. The method of claim 1 wherein the decoding tags are detected by determining the presence, amount, presence and amount, or absence of the decoding tags.

34. The method of claim 1 wherein the decoding tags are peptide nucleic acids, wherein each decoding tag has a different mass.

35. The method of claim 34 wherein each reporter tag is an oligonucleotide, and wherein each decoding tag has the same number of nucleotide bases complementary to the reporter tag.

36. The method of claim 35 wherein each decoding tag comprises a different number of 8-amino-3,6-dioxaoctanoic monomers.

37. The method of claim 34 wherein the decoding tags are detected by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy.

38. The method of claim 1 wherein the decoding tags are fluorescently-labeled oligonucleotides, wherein each decoding tag has a different combination of length and fluorescent label.

39. The method of claim 38 wherein each reporter tag is an oligonucleotide, and wherein each decoding tag has the same number of nucleotides complementary to the reporter tag.

40. The method of claim 39 wherein each decoding tag that has the same fluorescent label has a different number of nucleotides not complementary to the reporter tag.

41. The method of claim 38 wherein the decoding tags are detected by microdissection of the target sample and electrophoresis of the microdissected samples.

42. The method of claim 1 wherein the target molecules are in one or more target samples, wherein the target molecules include different modification states of the same target molecules, wherein the modifications are fragmentation, cleavage, phosphorylation, glycosylation, methylation, alkylation, dimerization, derivatization, depurination, conformation, or ribosylation.

43. The method of claim 42 wherein the target molecules include different phosphorylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different phosphorylation state, and wherein detection of the target molecules in the target sample is indicative of the phosphorylation state of the proteins in the target sample.

44. The method of claim 42 wherein the target molecules include different glycosylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different glycosylation state, and wherein detection of the target molecules in the target sample is indicative of the glycosylation state of the proteins in the target sample.

45. The method of claim 42 wherein the target molecules include different poly-ADP ribosylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different poly-ADP ribosylation state, and wherein detection of the target molecules in the target sample is indicative of the poly-ADP ribosylation state of the proteins in the target sample.

46. The method of claim 42 wherein the target molecules include different fragments of the same proteins, wherein each of the reporter molecules interacts with a different fragment, and wherein detection of the target molecules in the target sample is indicative of the fragments of the proteins in the target sample.

47. The method of claim 42 wherein the target molecules include different conformational states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different conformational state, and wherein detection of the target molecules in the target sample is indicative of the conformational state of the proteins in the target sample.

48. The method of claim 47 wherein at least one of the target molecules is a prion protein, wherein the conformation states include the prion conformation of a protein and the non-prion conformation of the protein.

49. The method of claim 1 wherein the pattern of the presence, amount, presence and amount, or absence of decoding tags constitutes a catalog of the target molecules.

50. The method of claim 49 wherein the target molecules are in two or more target samples, wherein the pattern of the presence, amount, presence and amount, or absence of decoding tags associated with each target sample constitutes a catalog of the target molecules in that target sample,

the method further comprising comparing one or more catalogs with one or more other catalogs.

51. The method of claim 1 wherein the target molecules, reporter molecules, or decoding tags are in an array, wherein each target molecule, reporter molecule, or decoding tag is immobilized at a different location in the array, and wherein detecting the decoding tags is accomplished by detecting the presence, amount, presence and amount, or absence of decoding tags in the arrays.

52. The method of claim 51 wherein the location, amount, or location and amount of decoding tags in the arrays constitutes a pattern of decoding tags in the arrays,

the method further comprising comparing the pattern of decoding tags in the arrays with the pattern of decoding tags in arrays determined in a separate procedure using a different one or more target molecules.

53. The method of claim 52 further comprising

comparing the pattern of decoding tags in the arrays with the pattern of decoding tags in arrays determined in a plurality of separate procedures using a plurality of different one or more target molecules.

54. The method of claim 1 wherein the target molecules are associated with cells, wherein the reporter molecules are associated with the target molecules, wherein each reporter molecule comprises a sorting tag, wherein the cells are sorted based on the sorting tags.

55. The method of claim 54 wherein the target molecules are cell surface proteins on cells, and wherein the reporter molecules are associated with the proteins on the cells.

56. The method of claim 1 wherein the decoding tags are separated and detected by high pressure liquid chromatography.

57. The method of claim 1 wherein each reporter molecule comprises a specific binding molecule, wherein the specific binding molecule is an antibody, a ligand, a binding protein, a receptor protein, a synthetic polyamide, a hapten, aptamer, carbohydrate, or an oligonucleotide.

58. The method of claim 57 wherein the binding protein is a DNA binding protein comprising one or more zinc finger motifs, leucine zipper motifs, helix-turn-helix motifs, or a combination.

59. The method of claim 1 wherein the target sample is immobilized, fixed, or adhered to a surface.

60. The method of claim 59 wherein the locations of the target molecules that are determined are the locations of the target molecules on the surface.

61. A method of detecting multiple target molecules in a sample, the method comprising

(a) bringing into contact four or more target samples and one or more reporter molecules,

wherein each target sample is brought into contact with a different set of reporter molecules, wherein the reporter molecules in each set of reporter molecules are different from the reporter molecules in the other sets of reporter molecules, wherein the reporter molecules are associated with target molecules in the target samples,

(b) mixing together four or more of the target samples,

(c) associating one or more decoding tags with the reporter molecules, wherein a different decoding tag corresponds to each different reporter molecule such that each decoding tag corresponds to only one of the target samples,

(d) detecting the decoding tags, wherein the decoding tags are disassociated from the reporter molecules during, or prior to, detection, wherein detecting the decoding tags indicates the presence of target molecules corresponding to the detected decoding tags, wherein detection of decoding tags corresponding to different target samples indicates the presence of the same target molecules in the corresponding target samples.

62. The method of claim 61 further comprising, prior to step (c),

amplifying the reporter molecules to produce multiple reporter tags for each reporter molecule, wherein the reporter tags remain associated with the reporter molecule, wherein the reporter tags produced from each reporter molecule are different from the reporter tags produced from other reporter molecules, wherein the decoding tags are associated with the reporter molecules by associating the decoding tags with the reporter tags.

63. The method of claim 62 wherein the reporter molecules are amplified using branched DNA.

64. The method of claim 63 wherein each reporter molecule comprises a reporter signal, wherein each branched DNA comprises multiple reporter tags and a tail which associates with the reporter signal.

65. The method of claim 62 wherein the reporter molecules are amplified using rolling circle amplification.

66. The method of claim 65 wherein each reporter molecule comprises a reporter signal, wherein the reporter signal primes rolling circle replication of an amplification target circle, wherein the amplification target circle comprises one or more sequences complementary to the reporter signal, wherein replication produces tandem sequence DNA, wherein the tandem sequence DNA contains a plurality of reporter tags.

67. The method of claim 62 wherein the reporter molecules are amplified using oligonucleotide dendrimers.

68. The method of claim 62 wherein each reporter molecule comprises a reporter signal, wherein each reporter molecule in the same set of reporter molecules

has the same reporter signal, wherein amplification of the reporter molecules is accomplished by amplifying the reporter signals.

69. The method of claim 61 wherein a plurality of decoding tags correspond to each target sample.

70. The method of claim 61 wherein the reporter molecules are associated with target molecules by covalently coupling the reporter molecules to the target molecules.

71. The method of claim 70 wherein the reporter molecules are associated with target molecules by incorporating the reporter molecules during synthesis of the target molecules.

72. The method of claim 71 wherein each reporter molecule comprises a primer portion and a reporter signal, wherein the primer portions prime synthesis of the target molecules, wherein the decoding tags are associated with the reporter molecules by associating the decoding tags with the reporter signals.

73. The method of claim 72 wherein the reporter signals are oligonucleotides, wherein the decoding tags are peptide nucleic acids that are complementary to the reporter signals.

74. The method of claim 70 wherein the reporter molecules are covalently coupled to the target molecules by reacting a reactive group on the reporter molecules with the target molecules.

75. The method of claim 61 wherein the decoding tags are oligonucleotides, carbohydrates, peptide nucleic acids, synthetic polyamides, antibodies, ligands, proteins, haptens, zinc fingers, aptamers, or mass labels.

76. The method of claim 61 wherein the target molecules are homing molecules, wherein, prior to step (a), the target samples or the source of the target samples are exposed to the homing molecules, wherein the presence of homing molecules corresponding to the detected decoding tags indicates the presence of molecules in the target samples to which the homing molecules are directed.

77. The method of claim 76 wherein the target samples are tissue samples, wherein, prior to step (a), the source of the tissue samples are exposed to the homing molecules, wherein the presence of homing molecules corresponding to the detected decoding tags indicates the presence of cells or molecules in the tissue samples to which the homing molecules are directed.

78. The method of claim 77 wherein the target molecules are tumor-homing peptides, wherein the presence of tumor-homing peptides corresponding to the detected decoding tags indicates the presence of tumor cells in the tissue samples.

79. The method of claim 77 wherein the organisms that are the source of the tissue samples are exposed to the homing molecules.

80. The method of claim 61 wherein the target samples are cells, wherein the cells have been sorted from other cells.

81. The method of claim 80 wherein the cells are sorted based on the presence, absence, or difference in amount of a cell marker.

82. The method of claim 61 wherein the target samples are organisms, wherein, following step (a), derivative target samples comprising the reporter molecules are prepared from the organisms.

83. The method of claim 61 wherein the target samples are tissues, wherein, following step (a), derivative target samples comprising the reporter molecules are prepared from the tissues.

84. The method of claim 61 wherein each decoding tag corresponds to a different reporter molecule, each reporter molecule corresponds to a different target molecule, or a combination.

85. The method of claim 84 wherein each decoding tag corresponds to a single reporter molecule, each reporter molecule corresponds to a single target molecule, or a combination.

86. The method of claim 84 wherein each decoding tag corresponds to multiple reporter molecules, each reporter molecule corresponds to multiple target molecules, or a combination.

87. The method of claim 61 further comprising, following step (b),

bringing into contact the mixed target samples and a capture array, wherein different target molecules become associated with different elements of the array, wherein the array elements with which the decoding tags are associated indicate the presence in the target samples of the target molecules corresponding to that array element.

88. The method of claim 87 wherein the capture array comprises capture tags, wherein each array element comprises a different capture tag.

89. The method of claim 87 wherein the capture tags are oligonucleotides, antibodies, haptens, ligands, or a combination.

90. The method of claim 87 wherein the capture array comprises a substrate.

91. The method of claim 90 wherein the substrate comprises beads, plates, or slides.

92. The method of claim 61 wherein at least two of the decoding tags are distinguished temporally via different fluorescent, phosphorescent, or chemiluminescent emission lifetimes.

93. The method of claim 61 wherein the decoding tags are detectable by nuclear magnetic resonance, electron paramagnetic resonance, surface enhanced raman scattering, surface plasmon resonance, fluorescence, phosphorescence, chemiluminescence, resonance raman, microwave, mass spectrometry, or a combination.

94. The method of claim 61 wherein the decoding tags are detected by mass spectrometry, electrophoresis, or chromatography.

95. The method of claim 61 wherein the decoding tags are peptide nucleic acids, wherein the decoding tags are detected by mass spectrometry, wherein the different decoding tags differ in mass.

96. The method of claim 95 wherein each reporter molecule comprises a reporter signal and a specific binding molecule, wherein the specific binding molecule of each reporter molecule interacts with a different target molecule, wherein the reporter signals are oligonucleotides, wherein the decoding tags are peptide nucleic acids that are complementary to the reporter signals.

97. The method of claim 61 wherein the decoding tags are detected by determining the presence, amount, presence and amount, or absence of the decoding tags.

98. The method of claim 61 wherein the decoding tags are peptide nucleic acids, wherein each decoding tag has a different mass.

99. The method of claim 98 wherein each reporter molecule comprises a reporter signal, wherein each reporter signal is an oligonucleotide, and wherein each decoding tag has the same number of nucleotide bases complementary to the reporter signal.

100. The method of claim 99 wherein each decoding tag comprises a different number of 8-amino-3,6-dioxaoctanoic monomers.

101. The method of claim 98 wherein the decoding tags are detected by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy.

102. The method of claim 61 wherein the decoding tags are fluorescently-labeled oligonucleotides, wherein each decoding tag has a different combination of length and fluorescent label.

103. The method of claim 102 wherein each reporter molecule comprises a reporter signal, wherein each reporter signal is an oligonucleotide, and wherein each decoding tag has the same number of nucleotides complementary to the reporter signal.

104. The method of claim 103 wherein each decoding tag that has the same fluorescent label has a different number of nucleotides not complementary to the reporter signal.

104. The method of claim 102 wherein the decoding tags are detected by microdissection of the target sample and electrophoresis of the microdissected samples.

106. The method of claim 61 wherein the decoding tags are zinc fingers.

107. The method of claim 61 wherein the target molecules are in one or more target samples, wherein the target molecules include different modification states of the same target molecules, wherein the modifications are fragmentation, cleavage, phosphorylation, glycosylation, methylation, alkylation, dimerization, derivatization, depurination, conformation, or ribosylation.

108. The method of claim 107 wherein the target molecules include different phosphorylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different phosphorylation state, and wherein detection of the target molecules in the target sample is indicative of the phosphorylation state of the proteins in the target sample.

109. The method of claim 107 wherein the target molecules include different glycosylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different glycosylation state, and wherein detection of the target molecules in the target sample is indicative of the glycosylation state of the proteins in the target sample.

110. The method of claim 107 wherein the target molecules include different poly-ADP ribosylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different poly-ADP ribosylation state, and wherein detection of the target molecules in the target sample is indicative of the poly-ADP ribosylation state of the proteins in the target sample.

111. The method of claim 107 wherein the target molecules include different fragments of the same proteins, wherein each of the reporter molecules interacts with a different fragment, and wherein detection of the target molecules in the target sample is indicative of the fragments of the proteins in the target sample.

112. The method of claim 107 wherein the target molecules include different conformational states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different conformational state, and wherein detection of the target molecules in the target sample is indicative of the conformational state of the proteins in the target sample.

113. The method of claim 112 wherein at least one of the target molecules is a prion protein, wherein the conformation states include the prion conformation of a protein and the non-prion conformation of the protein.

114. The method of claim 61 wherein the pattern of the presence, amount, presence and amount, or absence of decoding tags constitutes a catalog of the target molecules.

115. The method of claim 114 wherein the target molecules are in two or more target samples, wherein the pattern of the presence, amount, presence and amount, or absence of decoding tags associated with each target sample constitutes a catalog of the target molecules in that target sample,

the method further comprising comparing one or more catalogs with one or more other catalogs.

116. The method of claim 61 wherein the target molecules, reporter molecules, or decoding tags are in an array, wherein each target molecule, reporter molecule, or decoding tag is immobilized at a different location in the array, and wherein detecting the decoding tags is accomplished by detecting the presence, amount, presence and amount, or absence of decoding tags in the arrays.

117. The method of claim 116 wherein the location, amount, or location and amount of decoding tags in the arrays constitutes a pattern of decoding tags in the arrays,

the method further comprising comparing the pattern of decoding tags in the arrays with the pattern of decoding tags in arrays determined in a separate procedure using a different one or more target molecules.

118. The method of claim 117 further comprising

comparing the pattern of decoding tags in the arrays with the pattern of decoding tags in arrays determined in a plurality of separate procedures using a plurality of different one or more target molecules.

119. The method of claim 61 wherein the target molecules are associated with cells, wherein the reporter molecules are associated with the target molecules, wherein each reporter molecule comprises a sorting tag, wherein the cells are sorted based on the sorting tags.

120. The method of claim 119 wherein the target molecules are cell surface proteins on cells, and wherein the reporter molecules are associated with the proteins on the cells.

121. The method of claim 61 wherein the decoding tags are separated and detected by high pressure liquid chromatography.

122. The method of claim 61 wherein each reporter molecule comprises a specific binding molecule, wherein the specific binding molecule is an antibody, a ligand, a binding protein, a receptor protein, a synthetic polyamide, a hapten, aptamer, carbohydrate, or an oligonucleotide.

123. The method of claim 122 wherein the binding protein is a DNA binding protein comprising one or more zinc finger motifs, leucine zipper motifs, helix-turn-helix motifs, or a combination.

124. The method of claim 61 wherein the target sample is immobilized, fixed, or adhered to a surface.

125. The method of claim 124 wherein the locations of the target molecules that are determined are the locations of the target molecules on the surface.

126. A method of detecting multiple target molecules in a sample, the method comprising

(a) bringing into contact one or more target samples and one or more reporter molecules, wherein the reporter molecules are associated with target molecules in the target samples,

(b) associating one or more decoding tags with the reporter molecules, wherein a different decoding tag corresponds to each different reporter molecule, wherein the decoding tags are not covalently coupled to the reporter molecules,

(c) detecting the decoding tags, wherein the decoding tags are disassociated from the reporter molecules during, or prior to, detection, wherein the decoding tags correspond to reporter molecules, wherein the reporter molecules correspond to target molecules, wherein detecting the decoding tags indicates the presence of reporter molecules corresponding to the detected decoding tags, wherein the presence of reporter molecules indicates the presence of target molecules corresponding to the reporter molecules.

127. The method of claim 126 wherein each reporter molecule comprises one or more specific binding molecules, wherein the specific binding molecule of each reporter molecule interacts with a different target molecule.

128. The method of claim 126 further comprising, prior to step (b),

amplifying the reporter molecules to produce multiple reporter tags for each reporter molecule, wherein the reporter tags remain associated with the reporter molecule, wherein the reporter tags produced from each reporter molecule are different from the reporter tags produced from other reporter molecules, wherein the decoding tags are associated with the reporter molecules by associating the decoding tags with the reporter tags.

129. The method of claim 128 wherein the reporter molecules are amplified using branched DNA.

130. The method of claim 129 wherein each reporter molecule comprises a reporter signal, wherein each branched DNA comprises multiple reporter tags and a tail which associates with the reporter signal.

131. The method of claim 128 wherein the reporter molecules are amplified using rolling circle amplification.

132. The method of claim 131 wherein each reporter molecule comprises a reporter signal, wherein the reporter signal primes rolling circle replication of an amplification target circle, wherein the amplification target circle comprises one or

more sequences complementary to the reporter signal, wherein replication produces tandem sequence DNA, wherein the tandem sequence DNA contains a plurality of reporter tags.

133. The method of claim 128 wherein the reporter molecules are amplified using oligonucleotide dendrimers.

134. The method of claim 126 wherein the decoding tags are oligonucleotides, carbohydrates, peptide nucleic acids, synthetic polyamides, antibodies, ligands, proteins, haptens, zinc fingers, aptamers, or mass labels.

135. The method of claim 126 wherein the target molecules are homing molecules, wherein, prior to step (a), the target samples or the source of the target samples are exposed to the homing molecules, wherein the presence of homing molecules corresponding to the detected decoding tags indicates the presence of molecules in the target samples to which the homing molecules are directed.

136. The method of claim 135 wherein the target samples are tissue samples, wherein, prior to step (a), the source of the tissue samples is exposed to the homing molecules, wherein the presence of homing molecules corresponding to the detected decoding tags indicates the presence of cells or molecules in the tissue samples to which the homing molecules are directed.

137. The method of claim 136 wherein the target molecules are tumor-homing peptides, wherein the presence of tumor-homing peptides corresponding to the detected decoding tags indicates the presence of tumor cells in the tissue samples.

138. The method of claim 136 wherein the organisms that are the source of the tissue samples are exposed to the homing molecules.

139. The method of claim 135 wherein the target samples are tissue samples, wherein the tissue samples are exposed to the homing molecules after the tissue samples are sectioned.

140. The method of claim 126 wherein the target samples are cells, wherein the cells have been sorted from other cells.

141. The method of claim 140 wherein the cells are sorted based on the presence, absence, or difference in amount of a cell marker.

142. The method of claim 126 wherein the target samples are organisms, wherein, following step (a), derivative target samples comprising the reporter molecules are prepared from the organisms.

143. The method of claim 126 wherein the target samples are tissues, wherein, following step (a), derivative target samples comprising the reporter molecules are prepared from the tissues.

144. The method of claim 143 wherein the derivative target samples are tissue sections prepared from the tissues.

145. The method of claim 126 wherein each decoding tag corresponds to a different reporter molecule, each reporter molecule corresponds to a different target molecule, or a combination.

146. The method of claim 145 wherein each decoding tag corresponds to a single reporter molecule, each reporter molecule corresponds to a single target molecule, or a combination.

147. The method of claim 145 wherein each decoding tag corresponds to multiple reporter molecules, each reporter molecule corresponds to multiple target molecules, or a combination.

148. The method of claim 126 further comprising, following step (b),

bringing into contact the target samples and one or more capture arrays, wherein different target molecules become associated with different elements of the array, wherein the array elements with which the decoding tags are associated indicate the presence in the target samples of the target molecules corresponding to that array element.

149. The method of claim 148 wherein the capture array comprises capture tags, wherein each array element comprises a different capture tag.

150. The method of claim 148 wherein the capture tags are oligonucleotides, antibodies, haptens, ligands, or a combination.

151. The method of claim 148 wherein the capture array comprises a substrate.

152. The method of claim 151 wherein the substrate comprises beads, plates, or slides.

153. The method of claim 126 wherein at least two of the decoding tags are distinguished temporally via different fluorescent, phosphorescent, or chemiluminescent emission lifetimes.

154. The method of claim 126 wherein the decoding tags are detectable by nuclear magnetic resonance, electron paramagnetic resonance, surface enhanced

raman scattering, surface plasmon resonance, fluorescence, phosphorescence, chemiluminescence, resonance raman, microwave, mass spectrometry, or a combination.

155. The method of claim 126 wherein the decoding tags are detected by mass spectrometry, electrophoresis, or chromatography.

156. The method of claim 126 wherein the decoding tags are peptide nucleic acids, wherein the decoding tags are detected by mass spectrometry, wherein the different decoding tags differ in mass.

157. The method of claim 156 wherein each reporter molecule comprises a reporter signal and a specific binding molecule, wherein the specific binding molecule of each reporter molecule interacts with a different target molecule, wherein the reporter signals are oligonucleotides, wherein the decoding tags are peptide nucleic acids that are complementary to the reporter signals.

158. The method of claim 157 wherein the specific binding molecules are oligonucleotides, wherein the specific binding molecules hybridize to the target molecules, wherein each of the specific binding molecules form a stem and loop when not hybridized to a target molecule, wherein the stem is disrupted when the specific binding molecules hybridize to the target molecules.

159. The method of claim 126 wherein the decoding tags are detected by determining the presence, amount, presence and amount, or absence of the decoding tags.

160. The method of claim 126 wherein the decoding tags are peptide nucleic acids, wherein each decoding tag has a different mass.

161. The method of claim 160 wherein each reporter molecule comprises a reporter signal, wherein each reporter signal is an oligonucleotide, and wherein each decoding tag has the same number of nucleotide bases complementary to the reporter signal.

162. The method of claim 161 wherein each decoding tag comprises a different number of 8-amino-3,6-dioxaoctanoic monomers.

163. The method of claim 160 wherein the decoding tags are detected by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy.

164. The method of claim 126 wherein the decoding tags are fluorescently-labeled oligonucleotides, wherein each decoding tag has a different combination of length and fluorescent label.

165. The method of claim 164 wherein each reporter molecule comprises a reporter signal, wherein each reporter signal is an oligonucleotide, and wherein each decoding tag has the same number of nucleotides complementary to the reporter signal.

166. The method of claim 165 wherein each decoding tag that has the same fluorescent label has a different number of nucleotides not complementary to the reporter signal.

167. The method of claim 164 wherein the decoding tags are detected by microdissection of the target sample and electrophoresis of the microdissected samples.

168. The method of claim 126 wherein the decoding tags are zinc fingers.

169. The method of claim 126 wherein the target molecules are in one or more target samples, wherein the target molecules include different modification states of the same target molecules, wherein the modifications are fragmentation, cleavage, phosphorylation, glycosylation, methylation, alkylation, dimerization, derivatization, depurination, conformation, or ribosylation.

170. The method of claim 169 wherein the target molecules include different phosphorylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different phosphorylation state, and wherein detection of the target molecules in the target sample is indicative of the phosphorylation state of the proteins in the target sample.

171. The method of claim 169 wherein the target molecules include different glycosylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different glycosylation state, and wherein detection of the target molecules in the target sample is indicative of the glycosylation state of the proteins in the target sample.

172. The method of claim 169 wherein the target molecules include different poly-ADP ribosylation states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different poly-ADP ribosylation

state, and wherein detection of the target molecules in the target sample is indicative of the poly-ADP ribosylation state of the proteins in the target sample.

173. The method of claim 169 wherein the target molecules include different fragments of the same proteins, wherein each of the reporter molecules interacts with a different fragment, and wherein detection of the target molecules in the target sample is indicative of the fragments of the proteins in the target sample.

174. The method of claim 169 wherein the target molecules include different conformational states of the same proteins, wherein each of the reporter molecules interacts with a different protein in a different conformational state, and wherein detection of the target molecules in the target sample is indicative of the conformational state of the proteins in the target sample.

175. The method of claim 174 wherein at least one of the target molecules is a prion protein, wherein the conformation states include the prion conformation of a protein and the non-prion conformation of the protein.

176. The method of claim 126 wherein the pattern of the presence, amount, presence and amount, or absence of decoding tags constitutes a catalog of the target molecules.

177. The method of claim 176 wherein the target molecules are in two or more target samples, wherein the pattern of the presence, amount, presence and amount, or absence of decoding tags associated with each target sample constitutes a catalog of the target molecules in that target sample,

the method further comprising comparing one or more catalogs with one or more other catalogs.

178. The method of claim 126 wherein the target molecules, reporter molecules, or decoding tags are in an array, wherein each target molecule, reporter molecule, or decoding tag is immobilized at a different location in the array, and wherein detecting the decoding tags is accomplished by detecting the presence, amount, presence and amount, or absence of decoding tags in the arrays.

179. The method of claim 178 wherein the location, amount, or location and amount of decoding tags in the arrays constitutes a pattern of decoding tags in the arrays,

the method further comprising comparing the pattern of decoding tags in the arrays with the pattern of decoding tags in arrays determined in a separate procedure using a different one or more target molecules.

180. The method of claim 179 further comprising

comparing the pattern of decoding tags in the arrays with the pattern of decoding tags in arrays determined in a plurality of separate procedures using a plurality of different one or more target molecules.

181. The method of claim 126 wherein the target molecules are associated with cells, wherein the reporter molecules are associated with the target molecules, wherein each reporter molecule comprises a sorting tag, wherein the cells are sorted based on the sorting tags.

182. The method of claim 181 wherein the target molecules are cell surface proteins on cells, and wherein the reporter molecules are associated with the proteins on the cells.

183. The method of claim 126 wherein the decoding tags are separated and detected by high pressure liquid chromatography.

184. The method of claim 126 wherein each reporter molecule comprises a specific binding molecule, wherein the specific binding molecule is an antibody, a ligand, a binding protein, a receptor protein, a synthetic polyamide, a hapten, aptamer, carbohydrate, or an oligonucleotide.

185. The method of claim 184 wherein the binding protein is a DNA binding protein comprising one or more zinc finger motifs, leucine zipper motifs, helix-turn-helix motifs, or a combination.

186. The method of claim 126 wherein the target sample is immobilized, fixed, or adhered to a surface.

187. The method of claim 186 wherein the locations of the target molecules that are determined are the locations of the target molecules on the surface.

188. A method of detecting multiple target molecules in a sample, the method comprising

(a) bringing into contact one or more target samples and one or more reporter carriers, wherein each reporter carrier comprises one or more specific binding molecules, a carrier, and a plurality of decoding tags associated with the carrier,

(b) detecting the decoding tags, wherein the decoding tags correspond to reporter carriers, wherein the reporter carriers correspond to target molecules, wherein detecting the decoding tags indicates the presence of reporter carriers corresponding to the detected decoding tags, wherein the presence of reporter carriers indicates the presence of target molecules corresponding to the reporter carriers.

189. The method of claim 188 wherein the carrier is a liposome.

190. The method of claim 188 wherein each reporter carrier comprises at least 1,000 decoding tags.

191. The method of claim 188 wherein the decoding tags are detected by mass spectrometry, electrophoresis, or chromatography.

192. The method of claim 188 wherein the carrier is a viral particle, wherein the decoding tags are viral proteins containing selenium-substituted methionine, wherein the decoding tags are detected by mass spectrometry, wherein different decoding tags are distinguished by selenium-based differences in mass.

193. The method of claim 188 wherein the specific binding molecules of each reporter carrier interacts with a different target molecule, wherein a different decoding tag corresponds to each different reporter carrier.

194. The method of claim 188 wherein the decoding tags are disassociated from the reporter carriers during, or prior to, detection.

195. The method of claim 188 wherein the target molecules are homing molecules, wherein, prior to step (a), the target samples or the source of the target samples are exposed to the homing molecules, wherein the presence of homing molecules corresponding to the detected decoding tags indicates the presence of molecules in the target samples to which the homing molecules are directed.

196. The method of claim 195 wherein the target samples are tissue samples, wherein, prior to step (a), the source of the tissue samples is exposed to the homing molecules, wherein the presence of homing molecules corresponding to the detected decoding tags indicates the presence of cells or molecules in the tissue samples to which the homing molecules are directed.

197. The method of claim 196 wherein the target molecules are tumor-homing peptides, wherein the presence of tumor-homing peptides corresponding to the detected decoding tags indicates the presence of tumor cells in the tissue samples.

198. The method of claim 196 wherein the organisms that are the source of the tissue samples are exposed to the homing molecules.

199. The method of claim 195 wherein the target samples are tissue samples, wherein the tissue samples are exposed to the homing molecules after the tissue samples are sectioned.

200. The method of claim 188 wherein the target samples are cells, wherein the cells have been sorted from other cells.

201. The method of claim 200 wherein the cells are sorted based on the presence, absence, or difference in amount of a cell marker.

202. The method of claim 188 wherein the target samples are organisms, wherein, following step (a), derivative target samples comprising the reporter carriers are prepared from the organisms.

203. The method of claim 188 wherein the target samples are tissues, wherein, following step (a), derivative target samples comprising the reporter carriers are prepared from the tissues.

204. The method of claim 203 wherein the derivative target samples are tissue sections prepared from the tissues.

205. The method of claim 188 wherein each decoding tag corresponds to a different reporter carrier, each reporter carrier corresponds to a different target molecule, or a combination.

206. The method of claim 205 wherein each decoding tag corresponds to a single reporter carrier, each reporter carrier corresponds to a single target molecule, or a combination.

207. The method of claim 205 wherein each decoding tag corresponds to multiple reporter carriers, each reporter carrier corresponds to multiple target molecules, or a combination.

208. The method of claim 188 further comprising, following step (a),

bringing into contact the target samples and one or more capture arrays, wherein different target molecules become associated with different elements of the array, wherein the array elements with which the decoding tags are associated indicate the presence in the target samples of the target molecules corresponding to that array element.

209. The method of claim 208 wherein the capture array comprises capture tags, wherein each array element comprises a different capture tag.

210. The method of claim 208 wherein the capture tags are oligonucleotides, antibodies, haptens, ligands, or a combination.

211. The method of claim 208 wherein the capture array comprises a substrate.

212. The method of claim 211 wherein the substrate comprises beads, plates, or slides.

213. The method of claim 188 wherein at least two of the decoding tags are distinguished temporally via different fluorescent, phosphorescent, or chemiluminescent emission lifetimes.

214. The method of claim 188 wherein the decoding tags are detectable by nuclear magnetic resonance, electron paramagnetic resonance, surface enhanced raman scattering, surface plasmon resonance, fluorescence, phosphorescence, chemiluminescence, resonance raman, microwave, mass spectrometry, or a combination.

215. The method of claim 188 wherein the decoding tags are detected by mass spectrometry, electrophoresis, or chromatography.

216. The method of claim 188 wherein the decoding tags are peptide nucleic acids, wherein the decoding tags are detected by mass spectrometry, wherein the different decoding tags differ in mass.

217. The method of claim 216 wherein the specific binding molecule of each reporter carrier interacts with a different target molecule, wherein the decoding tags are peptide nucleic acids.

218. The method of claim 188 wherein the decoding tags are detected by determining the presence, amount, presence and amount, or absence of the decoding tags.

219. The method of claim 188 wherein the decoding tags are peptide nucleic acids, wherein each decoding tag has a different mass.

220. The method of claim 219 wherein each decoding tag comprises a different number of 8-amino-3,6-dioxaoctanoic monomers.

221. The method of claim 219 wherein the decoding tags are detected by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy.

222. The method of claim 188 wherein the decoding tags are fluorescently-labeled oligonucleotides, wherein each decoding tag has a different combination of length and fluorescent label.

223. The method of claim 222 wherein the decoding tags are detected by microdissection of the target sample and electrophoresis of the microdissected samples.

224. The method of claim 188 wherein the decoding tags are zinc fingers.

225. The method of claim 188 wherein the target molecules are in one or more target samples, wherein the target molecules include different modification states of the same target molecules, wherein the modifications are fragmentation, cleavage, phosphorylation, glycosylation, methylation, alkylation, dimerization, derivatization, depurination, conformation, or ribosylation.

226. The method of claim 225 wherein the target molecules include different phosphorylation states of the same proteins, wherein each of the specific binding molecules interacts with a different protein in a different phosphorylation state, and wherein detection of the target molecules in the target sample is indicative of the phosphorylation state of the proteins in the target sample.

227. The method of claim 225 wherein the target molecules include different glycosylation states of the same proteins, wherein each of the specific binding molecules interacts with a different protein in a different glycosylation state, and wherein detection of the target molecules in the target sample is indicative of the glycosylation state of the proteins in the target sample.

228. The method of claim 225 wherein the target molecules include different poly-ADP ribosylation states of the same proteins, wherein each of the specific binding molecules interacts with a different protein in a different poly-ADP ribosylation state, and wherein detection of the target molecules in the target sample is indicative of the poly-ADP ribosylation state of the proteins in the target sample.

229. The method of claim 225 wherein the target molecules include different fragments of the same proteins, wherein each of the specific binding molecules interacts with a different fragment, and wherein detection of the target molecules in the target sample is indicative of the fragments of the proteins in the target sample.

230. The method of claim 225 wherein the target molecules include different conformational states of the same proteins, wherein each of the specific binding

molecules interacts with a different protein in a different conformational state, and wherein detection of the target molecules in the target sample is indicative of the conformational state of the proteins in the target sample.

231. The method of claim 230 wherein at least one of the target molecules is a prion protein, wherein the conformation states include the prion conformation of a protein and the non-prion conformation of the protein.

232. The method of claim 188 wherein the pattern of the presence, amount, presence and amount, or absence of decoding tags constitutes a catalog of the target molecules.

233. The method of claim 232 wherein the target molecules are in two or more target samples, wherein the pattern of the presence, amount, presence and amount, or absence of decoding tags associated with each target sample constitutes a catalog of the target molecules in that target sample,

the method further comprising comparing one or more catalogs with one or more other catalogs.

234. The method of claim 188 wherein the target molecules or reporter carriers are in an array, wherein each target molecule or reporter carrier is immobilized at a different location in the array, and wherein detecting the decoding tags is accomplished by detecting the presence, amount, presence and amount, or absence of decoding tags in the arrays.

235. The method of claim 234 wherein the location, amount, or location and amount of decoding tags in the arrays constitutes a pattern of decoding tags in the arrays,

the method further comprising comparing the pattern of decoding tags in the arrays with the pattern of decoding tags in arrays determined in a separate procedure using a different one or more target molecules.

236. The method of claim 235 further comprising

comparing the pattern of decoding tags in the arrays with the pattern of decoding tags in arrays determined in a plurality of separate procedures using a plurality of different one or more target molecules.

237. The method of claim 188 wherein the target molecules are associated with cells, wherein the reporter carriers are associated with the target molecules,

wherein each reporter carrier comprises a sorting tag, wherein the cells are sorted based on the sorting tags.

238. The method of claim 237 wherein the target molecules are cell surface proteins on cells, and wherein the reporter carriers are associated with the proteins on the cells.

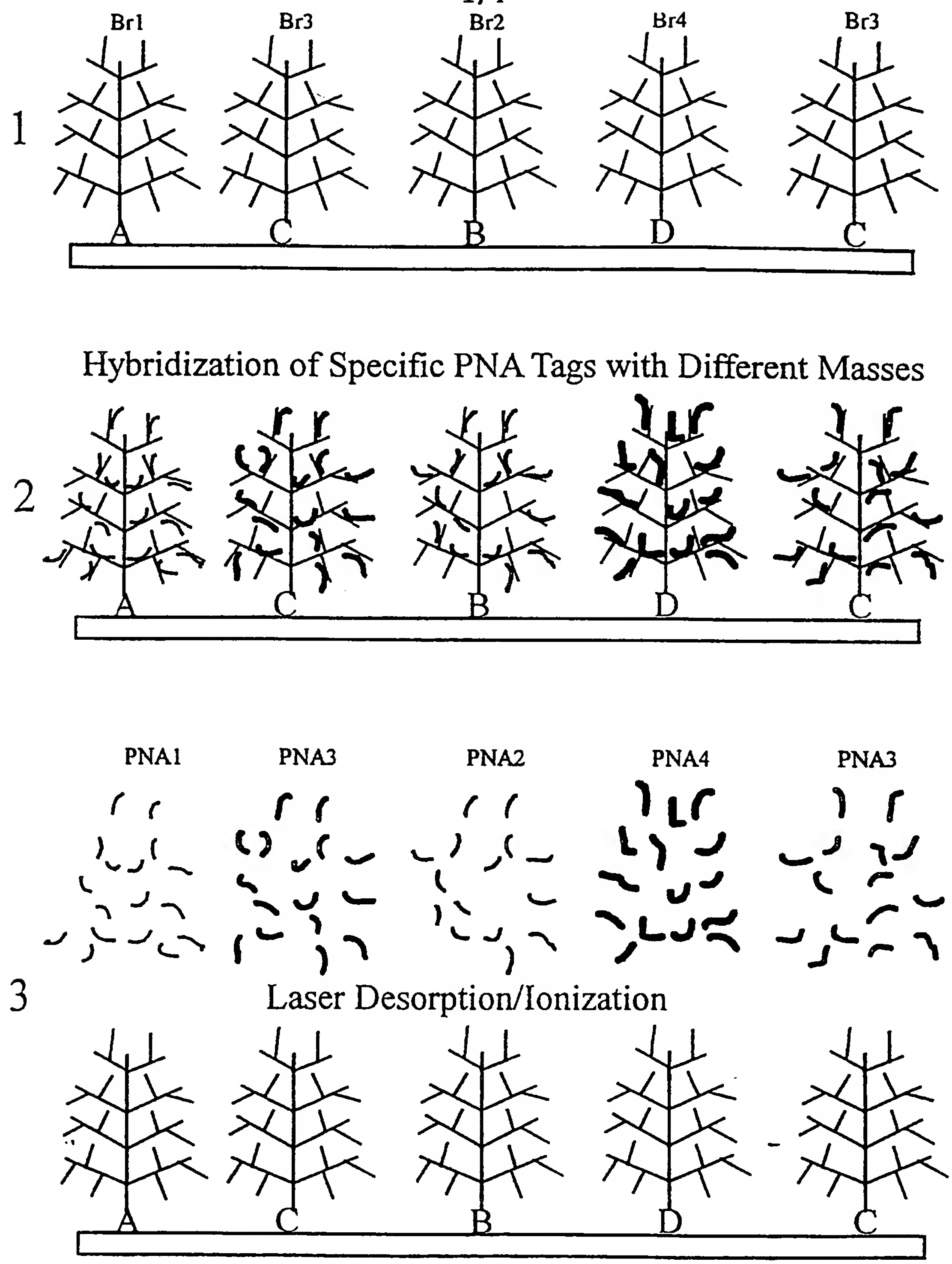
239. The method of claim 188 wherein the decoding tags are separated and detected by high pressure liquid chromatography.

240. The method of claim 188 wherein the specific binding molecule is an antibody, a ligand, a binding protein, a receptor protein, a synthetic polyamide, a hapten, aptamer, carbohydrate, or an oligonucleotide.

241. The method of claim 240 wherein the binding protein is a DNA binding protein comprising one or more zinc finger motifs, leucine zipper motifs, helix-turn-helix motifs, or a combination.

242. The method of claim 188 wherein the target sample is immobilized, fixed, or adhered to a surface.

243. The method of claim 242 wherein the locations of the target molecules that are determined are the locations of the target molecules on the surface.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Br3
Br2
Br4
Br3
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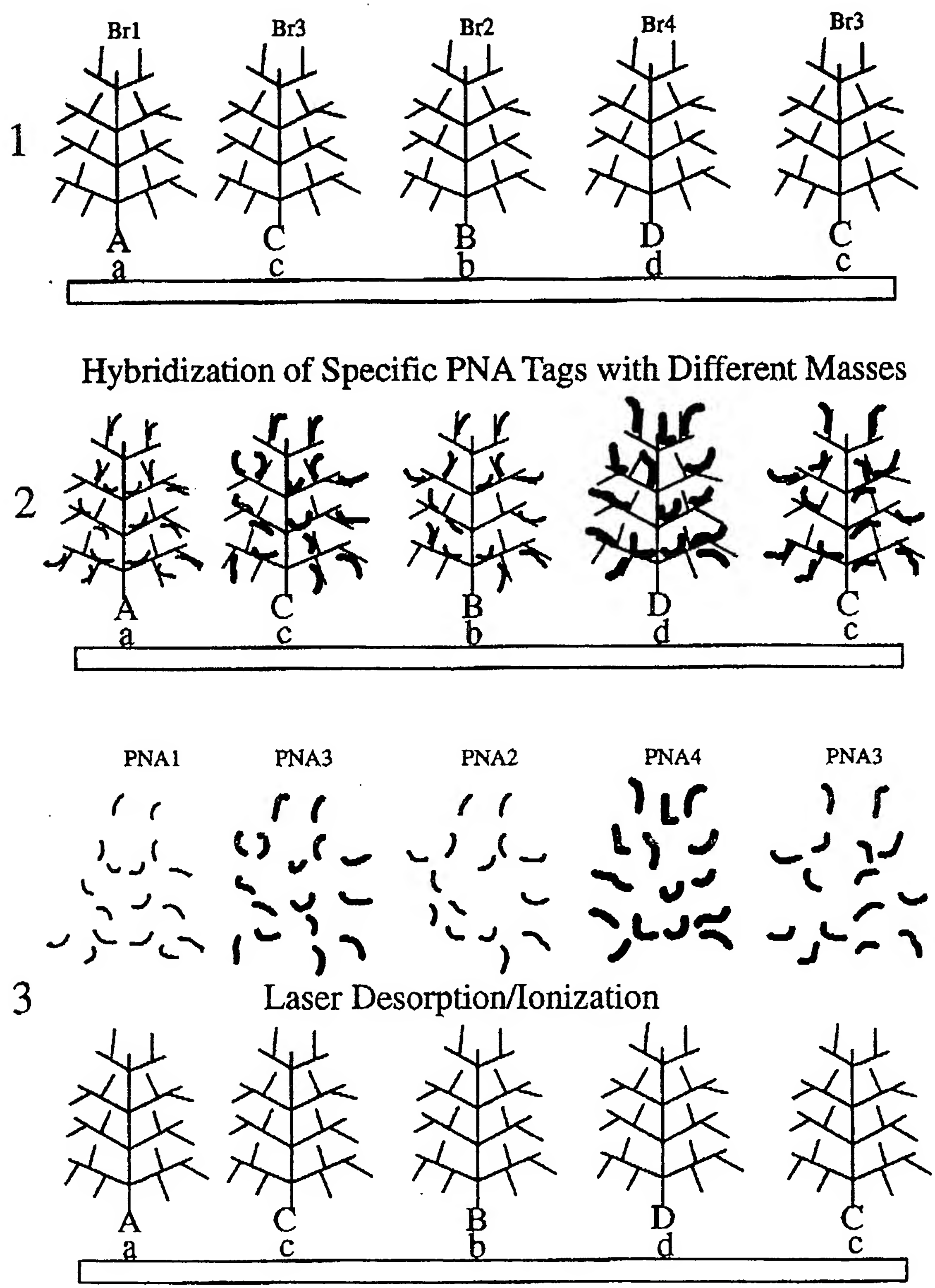
A
C
B
D
C
Hybridization of Specific PNA Tags with Different Masses
2
A
C
B
D
C
PNA1
PNA3
PNA2
PNA4
PNA3
3
Laser Desorption/Ionization
A
C
B
D
C

Figure 1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Br3
Br2
Br4
Br3
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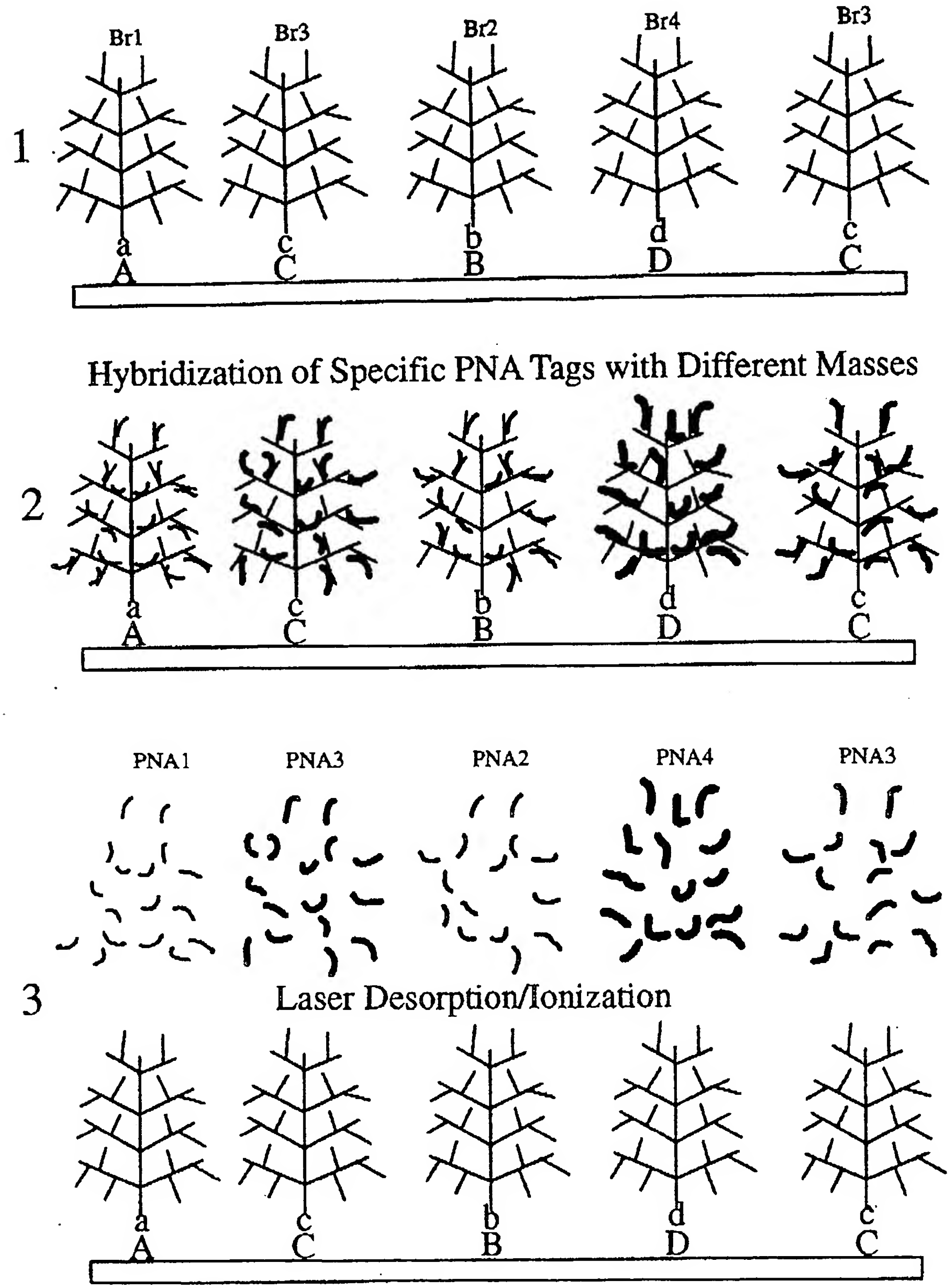
A a
C c
B b
D d
C c
Hybridization of Specific PNA Tags with Different Masses
2
A a
C c
B b
D d
C c
PNA1
PNA3
PNA2
PNA4
PNA3
3
Laser Desorption/Ionization
A a
C c
B b
D d
C c

Figure 2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Br3
Br2
Br4
Br3
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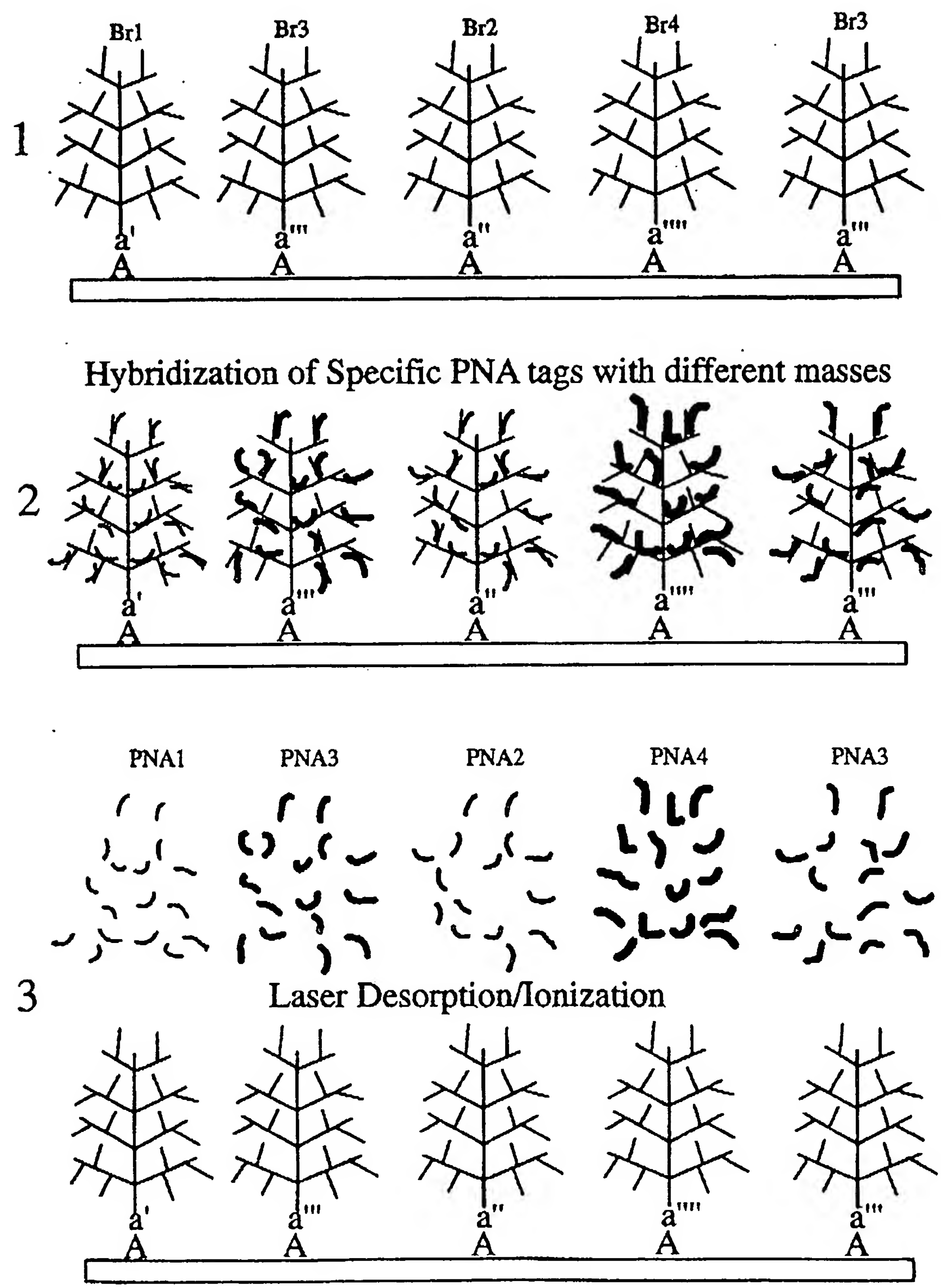
A
c
C
b
B
d
D
c
C
Hybridization of Specific PNA Tags with Different Masses
2
a
A
c
C
b
B
d
D
c
C
PNA1
PNA3
PNA2
PNA4
PNA3
3
Laser Desorption/Ionization
a
A
c
C
b
B
d
D
c
C

Figure 3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Br3
Br2
Br4
Br3
1
a'
a'''
a''
a''''
a'''
A
Hybridization of Specific PNA tags with different masses
2
PNA1
PNA3
PNA2
PNA4
PNA3
3
Laser Desorption/Ionization

Figure 4